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**MIGRATION OF HUMAN TYMPANIC  
EPITHELIUM  
IN  
HEALTH AND DISEASE**

**A THESIS SUBMITTED FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY**

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**THESIS CONTAINS  
VIDEO**

## ABSTRACT

**Boxall JD: *Migration of human tympanic membrane epithelium in health and disease.*** The gradual *en masse* migration of keratinised stratified squamous epithelium across the external surface of the human ear drum is an essential function in the maintenance of this delicate membrane by removing dead squamous epithelium to the external auditory meatus for dispersal. The abnormal presence of the same epithelium in the middle ear space produces a proliferating, expanding mass which cannot escape from its enclosed environment. This condition, described as *cholesteatoma\**, can, in turn, invade the facial nerve, inner ear and intracranial contents and require surgical intervention which in itself poses some risk to the individual.

The probable origins of cholesteatoma have been debated for more than a century.

Two experiments are presented (1) to examine the morphology and structure of a whole, normal tympanic membrane and (2) to examine cultures of epithelium dissected from the neck of a cholesteatoma sac for differences in morphology and migration from those found in tympanic membrane epithelium, using light microscopy and time-lapse video recordings.

The results show: (1) that the epithelial and fibroblast content can be determined in different layers of the whole tympanic membrane and that the close proximity and shared polarity between epithelium on the surface of the tympanic membrane and fibroblasts in the fibrous layer did not influence the direction of epithelial migration, (2) that in tissue culture, there are (a) marked

differences in the yield of epithelium from explants dissected from the neck of a cholesteatoma sac, (b) that these cultures produce epithelium with morphological differences to tympanic membrane epithelium and (c) that fibroblasts affect the fate of epithelium during tissue culture.

\*cholesteatoma, first described by Cruveilhier (1829) as a pearly tumour of the temporal bone and a few years later (1838) by Johannes Muller.



**Dedicated to the memory of Tony Frohlich, Wilfred Sankarsingh  
and all the backroom staff, engaged in scientific research  
and the understanding of human pathology.**

I am indebted to Professor Anthony Wright, under whose supervision this work was carried out and Dr Anna Furth for introducing me to the biological aspects of *en masse* migration. To Dr. David Proops for his cordiality and introduction to cholesteatoma pathology and to Professors Imrich Friedmann and Leslie Michaels whose expertise in Histopathology has always been an inspiration. To Dr. Simon Boxall, Oceanographer, for the loan of computer software. To nursing, medical, scientific, Peter Swartz and Dr. Peter Pearson - Librarians, Graham Neville, Ray Allen - Audio-visual, Andrew Gardner - Photography and many other staff at the Institute of Laryngology and Otology, U.C.L. and the Royal National Throat Nose and Ear Hospital for their generous, voluntary assistance and advice.

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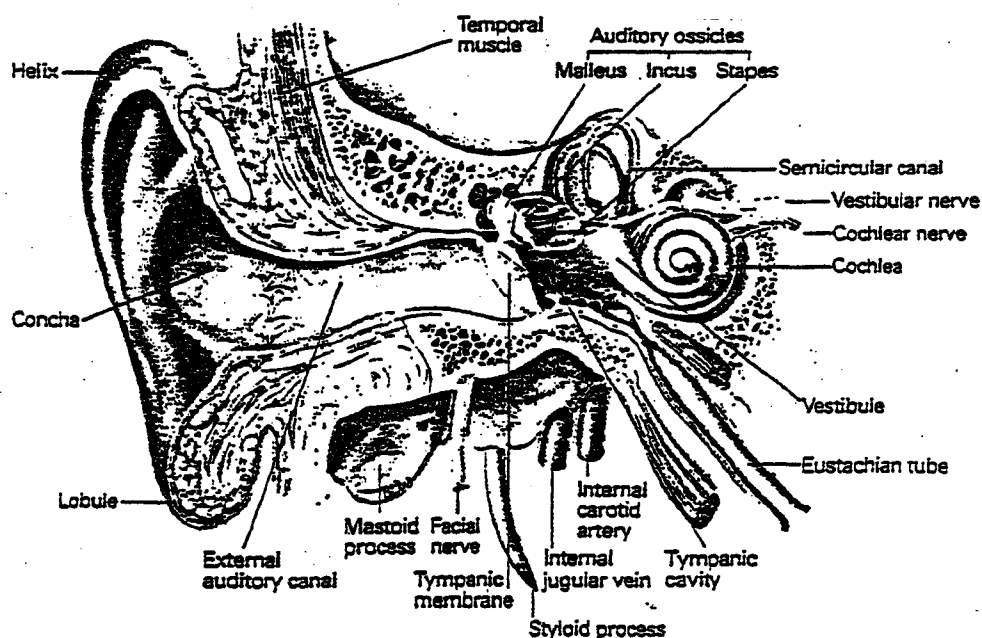
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## CHAPTER I : INTRODUCTION

### 1.1.1 A BRIEF ANATOMY OF THE EAR

The ear is conventionally and conveniently described in three parts: the outer, the middle and the inner ears (Fig. No. 1.1.1). The outer ear comprises the cartilaginous pinna and the external ear canal down to the level of the ear drum or tympanic membrane (TM). The middle ear comprises the TM and the tympanic cavity which contains the three ossicles - malleus, incus and stapes which connect the TM to the inner ear. The middle ear is gas containing and is connected to the nose by the Eustachian tube. It also extends backwards into the air filled spaces of the mastoid bone. The inner ear resides in the dense petrous temporal bone and comprises a system of canals and cavities, the labyrinth, which contain the specialised sensory cells of hearing and balance.

FIG.No. 1.1.1 Representation of the external, middle, internal ear-coronal plane:



LFRIEDMANN & W.ARNOLD: PATHOLOGY OF THE EAR Pub:Churchill Livingstone

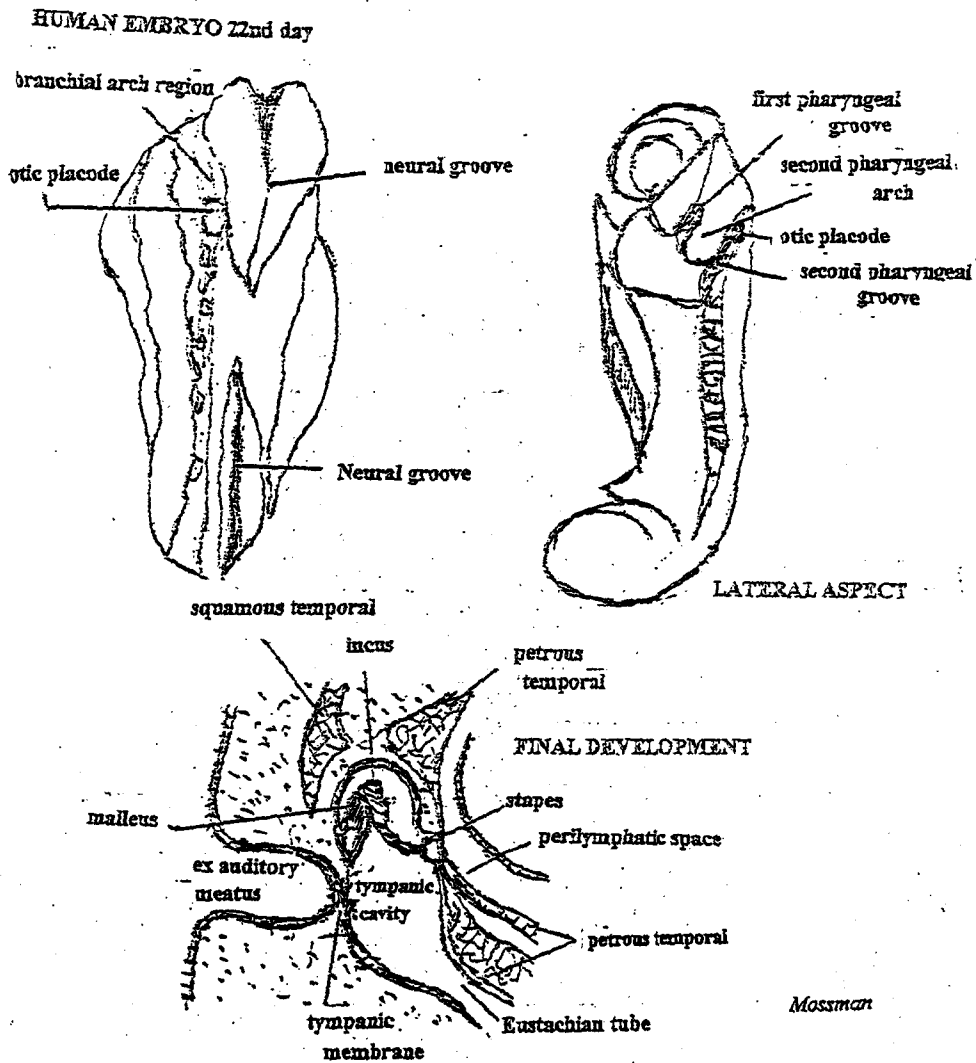
### 1.1.2 OUTER AND MIDDLE EAR: EMBRYOLOGY (Fig 1.1.2)

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At the head end of the developing mammalian embryo a series of band-like thickenings arise in the tissues surrounding each side of the primitive foregut or pharynx. These are called the branchial arches and there are usually five pairs. Each arch has a nerve and artery associated with it. The nerve of the first arch is the Vth Cranial Nerve (Trigeminal). The nerve of the 2nd arch is the VIIth Cranial Nerve (Facial) and of the 3rd arch the IXth, Glossopharyngeal. From each arch a small branch runs forward to the arch in front. The branch from the 2nd to the 1st arch is called the Chorda tympani. Between each arch develops a cleft on the outside surface (ectoderm) of the embryo and a pouch from the inside, pharyngeal surface (endoderm).

The external auditory meatus arises from a restricted portion of the first ectodermal cleft. The other ectodermal clefts usually regress although they occasionally persist as external pits or sinuses. The tympanic or middle ear cavity, the mastoid cavity and Eustachian tube is derived from the first endodermal pharyngeal pouch in the early embryo with small contributions from the dorsal portions of the second and third endodermal pouches and pharyngeal wall. The first pouch expands as the first cleft deepens and the two come into contact. The junction of the first pharyngeal pouch, with the first ectodermal cleft never breaks down in reptiles, birds or mammals but remains

Fig.No 1.1.2: OUTER AND MIDDLE EAR EMBRYOLOGY



intact and becomes the tympanic membrane, as a layer of tissue (mesenchyme) grows between the ectodermal and endodermal layers.

The thickenings which form the 1st and 2nd branchial arches develop into several structures including cartilage models of the future ossicles. The first arch become the malleus and incus whilst the second become the arch of stapes.

The formation of the tympanic cavity results in its epithelium gradually enveloping the ossicles, their tendons and ligaments and the chorda tympani nerve, so that all these structures receive a more or less complete epithelial investment. The TM possesses a superficial ectodermal layer, a deep endodermal layer and between these, a layer of mesenchyme which constitutes its lamina propria (see below).

During the tenth week of the development, posterior expansion of the tympanic cavity gives rise to the mastoid antrum. Its subsequent pneumatization together with the development of the mastoid air cells and mastoid process results, in part, from resorption of the mesenchymatous tissue filling these spaces. The tympanic cavity, which is filled by thickened mucosa and amniotic fluid during fetal life, is ventilated when pulmonary respiration begins. The middle ear space, the TM, the ossicles, the inner ear and mastoid antrum are all full, adult size at birth. There is however growth of the mastoid air cell system post-natally but even this has reached 90% of adult size by the age of five or six years.



**The mature mastoid air cell system is lined with fibrous tissue covered by a very thin layer of flat endothelium-like cells. In the middle ear, ciliated secretory epithelium is present near the orifice of the Eustachian tube and this mucosa is continuous with the mucous membrane of the nasopharynx by way of the Eustachian tube. Mucous is cleared from the middle ear via the Eustachian tube to the nasopharynx by the mucociliary clearance mechanisms.**

The external wall of the tympanic cavity (middle ear) is formed by the tympanic membrane, separating it from the external ear. The membrane is thin (0.1 mm), of elliptical shape, 8-9 mm X 9-10 mm (posterosuperior to anteroinferior), facing downwards and forwards, forming an angle of 55 degrees with the floor of the meatus in the adult. Overall, the membrane is concave towards the meatus and at the depth of the concavity is a small depression, the umbo, produced by the firm attachment of the handle of the malleus on the inner surface.

Most of the circumference of the membrane is thickened to form a cartilaginous ring, the tympanic annulus, which sits in a groove in the tympanic bone, the tympanic sulcus. The sulcus does not extend to the roof of the canal which is formed by part of the squamous bone. From the superior limits of the sulcus, the annulus becomes a fibrous band which runs centrally, as anterior and posterior malleolar folds. Above these, the upper fifth of the membrane, the pars flaccida, is a lax triangular region without a tympanic annulus. The majority of the membrane, the pars tensa, lies inferiorly to the pars flaccida and is taut and convex between its attachment to the malleus handle and the sulcus.

The tympanic membrane has three layers of tissue: epidermis, lamina propria and mucosa (Table 1 )

1. The external epidermis. This is continuous with the skin of the external meatus, and consists of four strata: The outermost stratum, *stratum corneum*, consists of several layers of non-viable keratinised epithelium with cell

membranes and desmosomes but no organelles. The second, *stratum granulosum*, containing keratohyaline and lamellar granules, some tonofilaments and interconnecting desmosomes. The third, *stratum spinosum*, consists of prickle cells with prominent interdigitations binding them to adjacent cells by desmosomes. These cells have a high nucleus to cytoplasm ratio, contain bundles of tonofilaments, mitochondria and ribosomes. The fourth is a single layer of cells, *stratum basal*, attached to the basement membrane which, apart from occasional cell prolongations, separates it from the lamina propria. None of these strata normally contain nerve endings or melanin granules. Mitotic figures can be seen in the basal and adjacent prickle cell layers in histological sections. As the daughter cells move towards the surface, there is continuing differentiation and keratinization as the cells die to become the stratum corneum on the external surface of the membrane. This layer is both protective and waterproof.

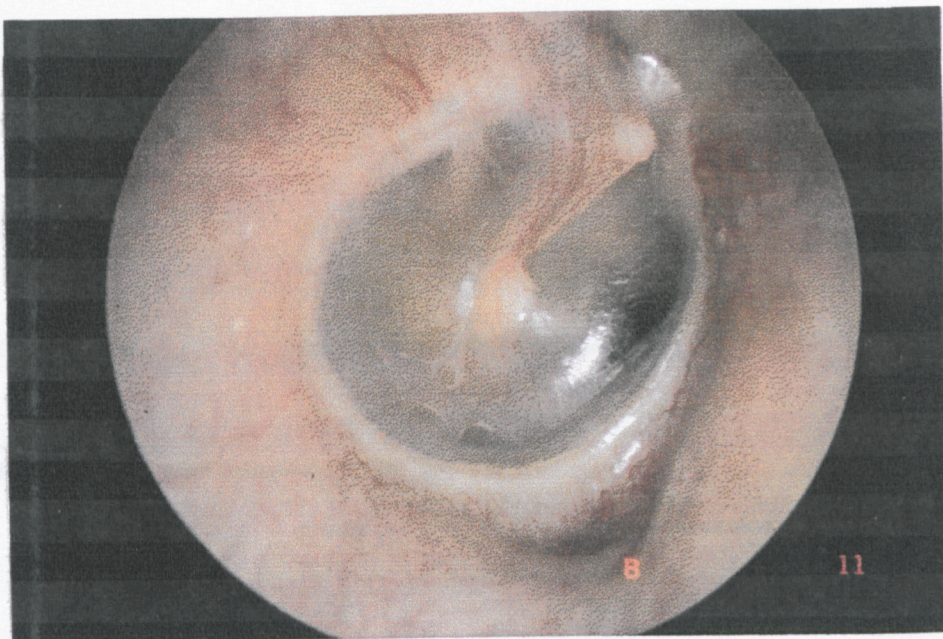
2. A middle fibrous layer, the lamina propria, containing collagen fibrils. In the pars tensa, the fibrils are usually in direct contact with the basement membrane, without the intervention of connective tissue. The lateral fibres, radial in orientation, are adjacent to deeper fibres arranged in circular, parabolic and transverse orientation.

3. A layer of mucosal epithelium, on a basement membrane, consists of simple squamous, cuboidal and pseudostratified cells with marked interdigitations and tight junctions between the apices facing the tympanic cavity. The exposed surface possesses numerous microvilli, granular secretory cells and patches of tall ciliated cells.

Between the deep layers of the lamina propria and inner mucosal layer, loose connective tissue contains fibroblasts, macrophages, capillaries and nerve fibres which are mostly unmyelinated.

The arterial blood supply to the tympanic membrane is complex. It arises from anastomotic branches supplying both the external auditory meatus and middle ear to feed a peripheral ring of vessels found in the connective tissue of the lamina propria and feed around the tip of the malleus handle. The venous drainage returns to the external jugular vein, transverse sinus, dural veins and venous plexus around the eustachian tube.

Fig. No 1.1.3: NORMAL TYMPANIC MEMBRANE



Normal right tympanic membrane. The circular translucent membrane is attached to the bony wall of the ear canal by a thick band of white fibrous tissue, called the annulus. The handle of the malleus runs from the centre of the membrane upwards and forwards to a prominent white bump called the lateral process. Above the lateral process is the "pars flaccida" portion of the membrane whilst below is the "pars tensa".

Table 1

STRUCTURE OF THE TYMPANIC MEMBRANE

External surface ↓ <b>EPITHELIAL LAYERS:</b> ↓	PARS TENSA	PARS FLACCIDA
<i>Stratum corneum:</i> ↓	several layers	several layers
<i>S.granulosum:</i> ↓	1-3 layers	up to 10 layers
<i>S.spinosum:</i> ↓	4-5 layers	4-5 layers
<i>S.basal:</i> ↓	1 layer	1 layer
↓	----- basement membrane -----	
↓	<b>FIBROBLAST AND COLLAGENOUS CORE:</b>	
↓		
<i>Connective tissue:</i> ↓	absent	thin layer
↓		
<i>Lamina propria:</i> ↓	connective tissue with capillaries	
↓		
<i>Lateral layer of radial collagenous fibres:</i> ↓	present	random orientation
↓		
<i>Medial layer of circular fibres:</i> ↓	present	random orientation
↓		
<i>Lamina propia:</i> ↓	connective tissue with capillaries	
↓		
<b>EPITHELIAL LINING OF THE MIDDLE EAR:</b>	single layer of cubical epithelium	

**“The external ear is a cul-de-sac which protects the delicate tympanic membrane, at the same time preventing the normal dispersal of epidermal keratin squames by exposure to the abrasive effect of moisture and air currents. In order to overcome this, nature has devised the means of their dispersal by continuous epithelial migration from the umbo to the periphery of the tympanic membrane and thence to the walls of the ear canal and external elements.”**

**The first mention that canal wall skin migrates to its periphery and that the same phenomenon occurs on the drumhead was made by Burnett (1877). His description of migration is printed above. These findings were complimented by Buck (1880) who proposed that this function explained the manner of clearing of cerumen (wax) from the ear. The direction of this epithelial migration was plotted by using small paper discs by Blake (1882) who also noted that the keratin layer of the deep canal became detached at the junction of the osseous and cartilagenous portions of the canal and (Blake, 1909) proposed that malfunction of migration caused keratitis obturans which is a condition where the canal skin accumulates and causes expansion of the bony canal wall. The self-clearing process of the external canal was again recognised by Bezold (1908) who unravelled a plug of epidermis from the external ear to find that it was several times longer than the canal.**

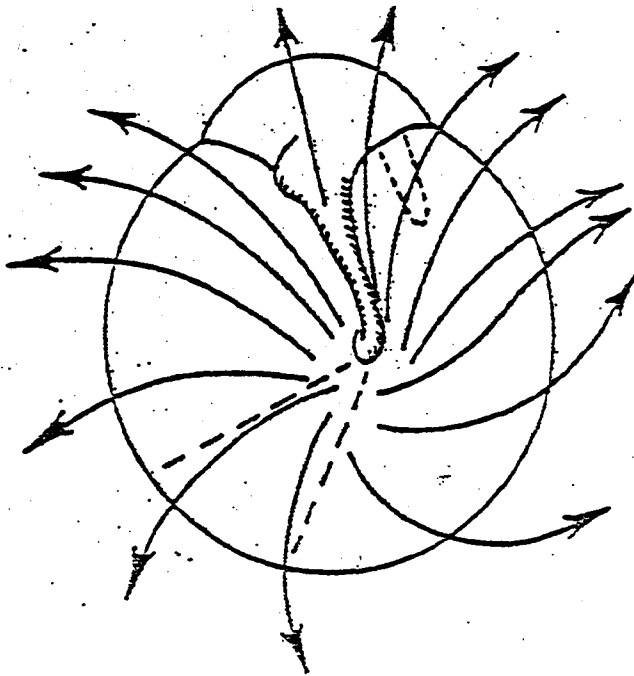
**An incidental observation by Stinson (1936), that a piece of reed accidentally embedded in the tympanic membrane was gradually carried from the drumhead to the posterior canal wall and then to the exterior, paved the way**

for his experiments using daubs of Indian ink, although none of these findings was recorded in any detail. Magnoni (1938) observed dispersal of ink across the membrane and reported that migration can only be from the drumhead to the canal wall and, therefore, cholesteatoma cannot arise from inward invasion.

Simonetta (1947) suggested that an accumulation of ceruminous plugs was due to failure in migration. Much of the research that followed during the 1950's was devoted to the problem of rejection of prostheses and the repair of perforations of the tympanic membrane by migrating epithelium.

Litton (1963), estimated the rate of migration as 0.05mm/d and suggested (1968) that the shape of the cells and cumulative effect of their leaving the basal layer accounted for their movement. Alberti, (1964), observed that migration originated in the basal layer of the epithelium and suggested that random mitosis in the lowest spinous layer produced cellular displacement. An accurate map of the direction of migration was prepared from extensive ink-dot trials on volunteer medical and dental students.

FIG 1.1.4:



Epithelial migration on the tympanic membrane.  
Alberti (1964)

The estimated migration 0.09 to 0.16mm/d was reported together with the finding that perforated tympanic membranes grafted with extra-auricular skin behaved in an entirely different way from drumhead skin. The rate of natural desquamation was much in excess of that of the drumhead which necessitated regular ear cleaning and, occasionally, cysts were formed.

Electron microscope studies of the guinea pig tympanic membrane by F.R. Johnson *et al.*, (1968) confirmed the lack of desmosomes and epithelial pegs which would assist migration and Lim (1972) found large irregular gaps between the basal and suprabasal cells.

Boedts (1978) using ink dots found that many of the dots initially remained stationary at the umbo before migrating across the tympanic annulus over 60-80 days ( 0.07-0.08 mm/day). Measurement of further migration as to the rate in the external auditory canal proved too inaccurate to support any valid conclusion. Ink dots stopped at perforations and at ventilation tubes, which had become embedded in keratin rests, mainly on the centripetal side where the epithelium desquamated in situ. Boedts considered that the migratory events occurred within the stratum corneum.

Proops *et al.*, (1984) demonstrated that senescent migratory skin resembled cholesteatoma in tissue culture.

A. Johnson *et al.*, (1984, 1986) took sections of skin from temporal bones to study the presence of wrinkles in the surface of the deep canal stratum corneum. The migrating sheet of stratum corneum is seen to wrinkle laterally in the axis of the canal when passing through the isthmus and transversely when it



encounters stationary hairs and ceruminous glands at the junction of the deep, superficial portion of the ear canal. This provides a mechanism for desquamation. In the guinea pig, the high actin content of basal cells together with their shape and orientation suggest migration in the basal or suprabasal layer, or both, with more desmosomes in the superficial layers compared with the deeper layers of the membrane. Ink particles introduced intracellularly into the guinea pig epidermis and underlying tissue are subsequently found in the form of a trail left behind a migrating scab in the upper dermis. This indicates that migration occurs in the deeper layers of the epidermis (with the possibility of a different rate of migration in different layers) and that migration stops at the junction of the deep and superficial parts of the canal.

Using a mass of ball bearings to represent tympanic membrane epithelium, Smelt & Hawke (1986) experimented by inserting small additional groups to represent mitoses and the arrival of new corneocytes, either at random to mimic Alberti's suggestion, or close to the centre at Litton's suggestion. They reported that both mechanisms produced the faithful representation of a central slow zone dispersion of ink dots on a drum.

Cecire et al., (1991) investigated whether migration is initiated by shedding at the chondro-osseous junction of the external ear canal and whether the periauricular muscles attached to this junction may exert a pulling effect to encourage epithelial migration. Using guinea pigs and ink dots, he obtained negative results.

On the surface of the tympanic membrane, keratin moves in geodesic lines i.e., the shortest distance on a hemisphere, between the umbo and the annulus.

The effect of the stratum corneum splitting up, as it covers an increasing surface area, is to produce thickened patchy ridges. In addition, the oblique position of the drum ( $55^{\circ}$ ) degrees from vertical, in relation to the bore of the external meatus, produces the optical illusion of these ridges appearing to form spiral curves (Weinberger *et al.*, 1986).

#### EPITHELIAL MIGRATION *IN VITRO*

Using tissue culture as a tool, Sakai *et al.*, (1982) reported *in vitro* differences between the migration of canal skin (0.5 - 1 mm/day) and cholesteatoma epithelium (0 - 0.3 mm/day). I used time-lapse video and recorded an average of 1 mm/day in cholesteatoma and tympanic membrane (Boxall *et al.*, 1988) and later (ibid. 1993) reported the movement of epithelial colonies to be intermittent. Specimens were prepared either by culturing explants of tympanic membrane epithelium to produce outgrowths or by trypsinising the tissue and culturing the epithelium as colonies on a feeder layer of irradiated mouse fibroblasts. Outgrowths from the explants emigrated en masse, in a linear direction, away from the explants after about ten days incubation. This time may be increased when epithelium needs to migrate and cascade down the side of an explant in order to reach a suitable substratum, e.g., coverslip, on which to propogate. Colonies growing from trypsinised epithelium, first rotated about their axes before migrating in a zig-zag and finally in a linear direction. During en masse migration, 80% of the mitoses are confined to the leading 25% of epithelium matrix producing a pull-push rhythm: a pulling effect from the advancing leading edge together with a pushing effect behind the ridge of keratinised epithelium (Boxall 1993). This ridge (Fig 1.1.5) consists of dead

keratinised cells swept back from the leading edge and sharing its polarity. The trailing cells show a five-fold increase in area as they are dragged forward and mitotic activity is scarce (personal observation).

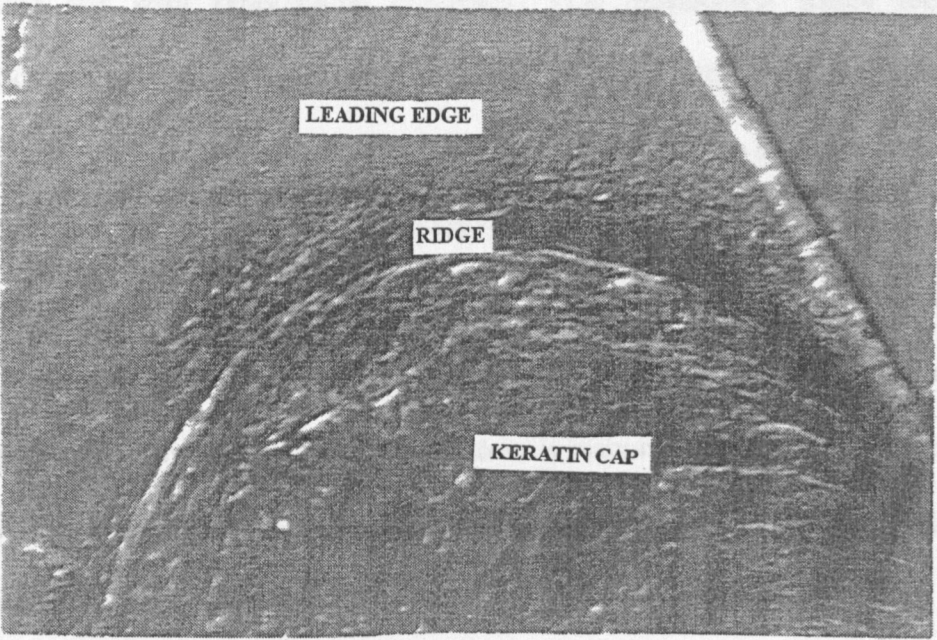
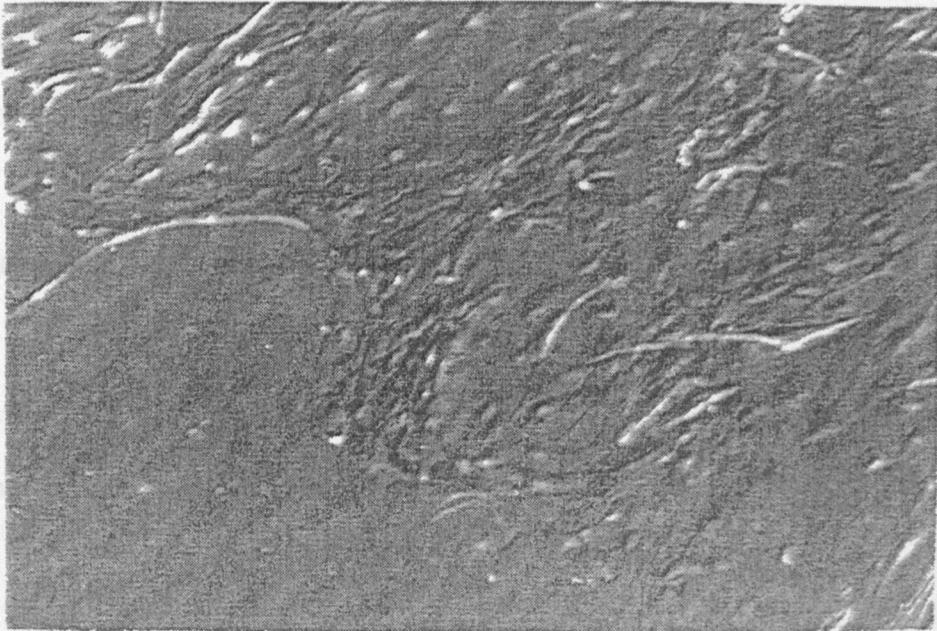


Fig 1.1.5: Tympanic membrane and cholesteatoma epithelium (except epithelium from the neck of a cholesteatoma sac) produces a ridge of keratinised epithelium behind the leading edge. There are several ridges on the right hand side where this *en masse* migrating epithelium has reached the edge of a coverslip and turned left through an angle of 90° (below:) The surface area of trailing cells increases as they are dragged forward. 100µm



Land living vertebrates that hear - reptiles, birds and mammals have developed a mechanism to convey air borne sound waves to the fluids of the inner ear with remarkable efficiency. This mechanism which technically attempts to match the impedance of air to that of the cochlear fluids is the middle ear which has been described in the preceeding chapters. The system comprises the taut but flexible tympanic membrane with a large surface area, and a system of levers suspended in air, thereby reducing any frictional losses, which connect the membrane to a small area in contact with the inner ear fluids. This impedance matching system is remarkably effective in that about 50% of the sound energy reaching the membrane is transferred to the inner ear.

However, the evolution of such a system has brought with it inherent problems which are common to biological gas pockets. These are:

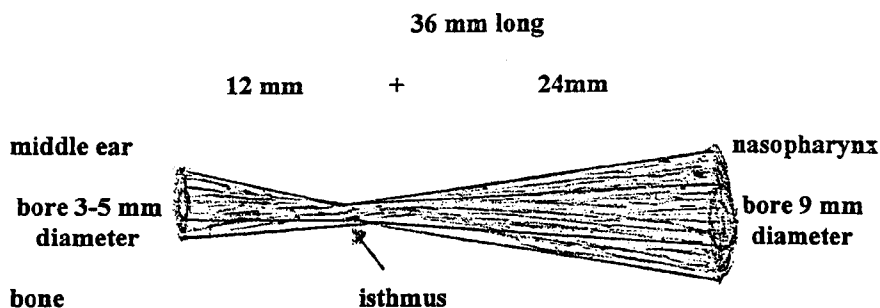
1. The need to maintain the gas pressure in the pocket at atmospheric in order to maintain the tympanic membrane in its unstretched, most efficient, sound collecting state.
2. The need to remove the debris, in terms of dead cells and fluids, that is produced by the cells lining this pocket.

These functions are performed by the Eustachian tube which passes from the anterior wall of the middle ear to the nasopharynx which in mammals is located at the back of the nasal cavities and above the soft palate.

In man, the Eustachian tube is about 36 mm long with the lateral third, which connects with the middle ear, having a solid bony wall. This portion is in effect part of the middle ear. The medial two thirds which connect with the nasopharynx, has flexible, fibro-cartilagenous walls with 2 muscles attached to the edges of the wall. There is a narrow region called the Isthmus between the two portions.

The floor of the Eustachian tube has a lining of ciliated epithelium with the ciliary beat propelling mucus from the middle ear towards the nasopharynx. In this way the debris and mucus that arises from the middle ear can be cleared provided air can travel from the nasopharynx to make up for the volume of mucus that has been removed.

Fig 1.1.6: EUSTACHIAN TUBE (scale X 2)



The maintainance of the middle ear pressure at, or close to atmospheric is, however more complex. Any “complex” gas is a mixture of various component gases and within that mixture each has its own partial pressure depending upon its concentration.

Thus in air the partial pressures of the 4 gases relevant to our discussion are:

Nitrogen	$p_{N_2}$	:	563 mm Hg
Oxygen	$p_{O_2}$	:	150 mm Hg
Carbon dioxide	$p_{CO_2}$	:	zero (approx)
Water vapour (in saturated air)	$p_{H_2O}$	:	47 mm Hg
Total pressure			<u>760 mm Hg</u>

After this gas mixture has been breathed into the lungs various exchanges take place with oxygen being preferentially absorbed and carbon dioxide excreted.

Arterial blood which has come into contact with the inhaled air in the lungs has a high  $p_{O_2}$  and a moderate  $p_{CO_2}$ .

The levels are:

#### ARTERIAL BLOOD

$p_{N_2}$	575 mm Hg
$p_{O_2}$	93 mm Hg
$p_{CO_2}$	39 mm Hg
$p_{H_2O}$	47 mm Hg
total	<u>754 mm Hg</u>

By the time this arterial blood has passed through the capillaries and has given up some of its oxygen to the tissues and in turn received carbon dioxide, the constituents of venous blood are:

**VENOUS BLOOD**

<b>pN<sub>2</sub></b>	<b>575 mm Hg</b>
<b>pO<sub>2</sub></b>	<b>38 mm Hg</b>
<b>pCO<sub>2</sub></b>	<b>44 mm Hg</b>
<b>pH<sub>2</sub>O</b>	<b>47 mm Hg</b>
<b>total</b>	<b><u>704 mm Hg</u></b>

The discrepancy in the overall pressures (pTotal 704 mm Hg in venous and 754 mm Hg in arterial blood), occurs because of the extra “solubility” of CO<sub>2</sub> as it is converted into bicarbonate by the enzyme carbonic anhydrase.

Nevertheless these are the partial pressures of the blood that is in very close contact with the gas of the middle ear.

The air which is present in the nasopharynx and which is available for transport to the middle ear, is expired air since swallowing occurs after breathing out. This air has the approximate partial pressures:

<b>pN<sub>2</sub></b>	<b>572 mm Hg</b>
<b>pO<sub>2</sub></b>	<b>100 mm Hg</b>
<b>pCO<sub>2</sub></b>	<b>41 mm Hg</b>
<b>pH<sub>2</sub>O</b>	<b>47 mm Hg</b>
<b>total</b>	<b><u>760 mm Hg</u></b>

The relationship between the available air and middle ear venous blood is thus:

<b>"AIR" AVAILABLE</b>		
	<b>TO MIDDLE EAR AIR</b>	<b>VENOUS BLOOD</b>
<b>pN<sub>2</sub></b>	<b>572 mm Hg</b>	<b>575 mm Hg</b>
<b>p O<sub>2</sub></b>	<b>100 mm Hg</b>	<b>38 mm Hg</b>
<b>pCO<sub>2</sub></b>	<b>41 mm Hg</b>	<b>44 mm Hg</b>
<b>pH<sub>2</sub>O</b>	<b>47 mm Hg</b>	<b>47 mm Hg</b>
<b>total</b>	<b>760 mm Hg</b>	<b>704 mm Hg</b>

If there was a free flow of gases across the membranes lining the middle ear then middle ear pressure would be lower than atmospheric by 56 mm Hg (760-704). This would result in a large pressure gradient across the tympanic membrane which would adversely affect its function.

This theoretical problem is overcome by a combination of the periodic supply of nasopharyngeal gas via the Eustachian tube and by the very slow diffusion rates of nitrogen so that the nitrogen "builds up" and its high partial pressure acts to maintain the overall pressure close to atmospheric.

Measured middle ear gas pressures are given below,

<b>pN<sub>2</sub></b>	<b>623 mm Hg</b>
<b>pO<sub>2</sub></b>	<b>40 mm Hg</b>
<b>pCO<sub>2</sub></b>	<b>50 mm Hg</b>
<b>pH<sub>2</sub>O</b>	<b>47 mm Hg</b>
<b>total</b>	<b>760 mm Hg</b>



The exact mechanism of Eustachian tube function and the causes of dysfunction are not really known and are outside the scope of this thesis. Suffice it to say that inadequate ventilation of the middle ear results in a negative middle ear pressure (relative to atmospheric) and changes in the tympanic membrane. This becomes indrawn or retracted and if the condition persists it appears that the middle fibrous layer of the membrane degenerates so that it becomes lax and floppy.

It is possible and indeed likely that intercurrent infections may contribute to this process of the thinning of the membrane. Repeated episodes of negative middle ear pressure lead to further retraction and the formation of retraction pockets.

Elnor (1976) calculated that under physiological and steady state conditions, about 1-2 ml of gas enter the middle ear every 24 hours. This amount equals the net amount of gas lost per day by diffusion from the middle ear cleft, through the mucosa into the blood in a normal ear.

While pressure fluctuations in the nasopharynx exist when we breath, only insignificant pressure fluctuations occur when we swallow, which is the period when the Eustachian tube opens. Thus, at a physiological steady state the main, if not the only pressure difference between the Eustachian tube and the nasopharynx, results from regular gas loss from the middle ear into the circulation. Thus, if we lose 1- 2 ml of gas per day into the circulation and swallow about 1,000 times per day, about 1-2 micro litres should be expected to be lost by diffusion every 1-2 minutes and to be regained by swallowing.

Therefore, if Eustachian tube function has an upper limit of gas transfer from the nasopharynx, then any process in the middle ear which removes gas at a greater rate than it can be supplied will result in a negative middle ear pressure and subsequent retraction of the tympanic membrane.

An element of this thesis (see Appendices) has been to explore whether bacteria in a closed system are capable of producing a reduction in ambient gas pressure. If they did, and if the effect was sufficiently great, then bacterial growth could become a significant, if not the major, factor in the development of retraction pockets and subsequently of cholesteatoma.

Partial and total pressures in mm Hg of air, blood and middle ear lumen  
gas at sea level:

	Air (37°C) saturated	Alveolar	Arterial	Mixed venous blood	Middle ear*
pO <sub>2</sub>	150	102	93	38	40
pCO <sub>2</sub>	0	39	39	44	50
pH <sub>2</sub> O	47	47	47	47	47
pN <sub>2</sub> **	563	572	575	575	623
	760	760	754	704	760

\*average multiple measurements

\*\* nitrogen and other inner gases

Dejours 1981

### 1.2.1 CHOLESTEATOMA: HISTORICAL REVIEW

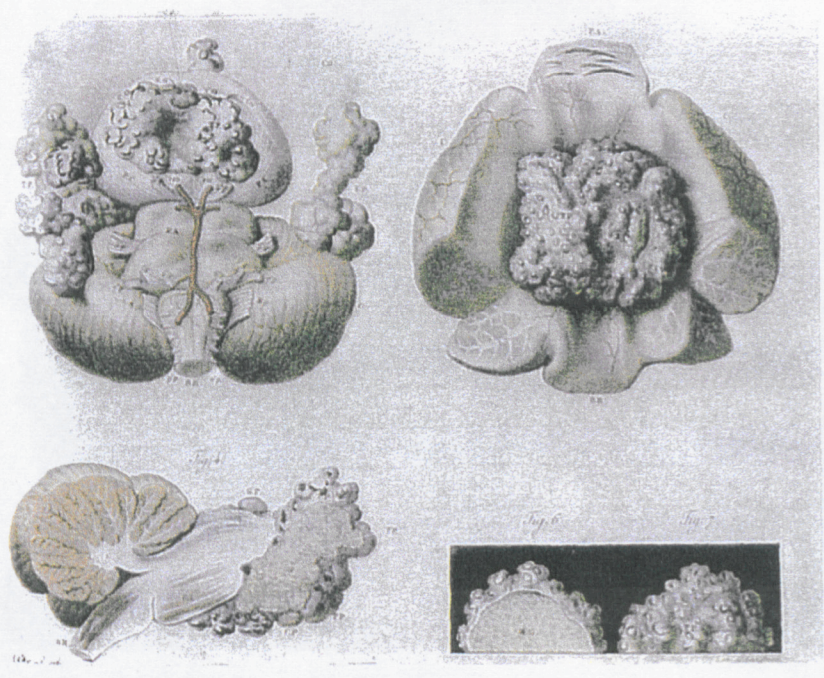
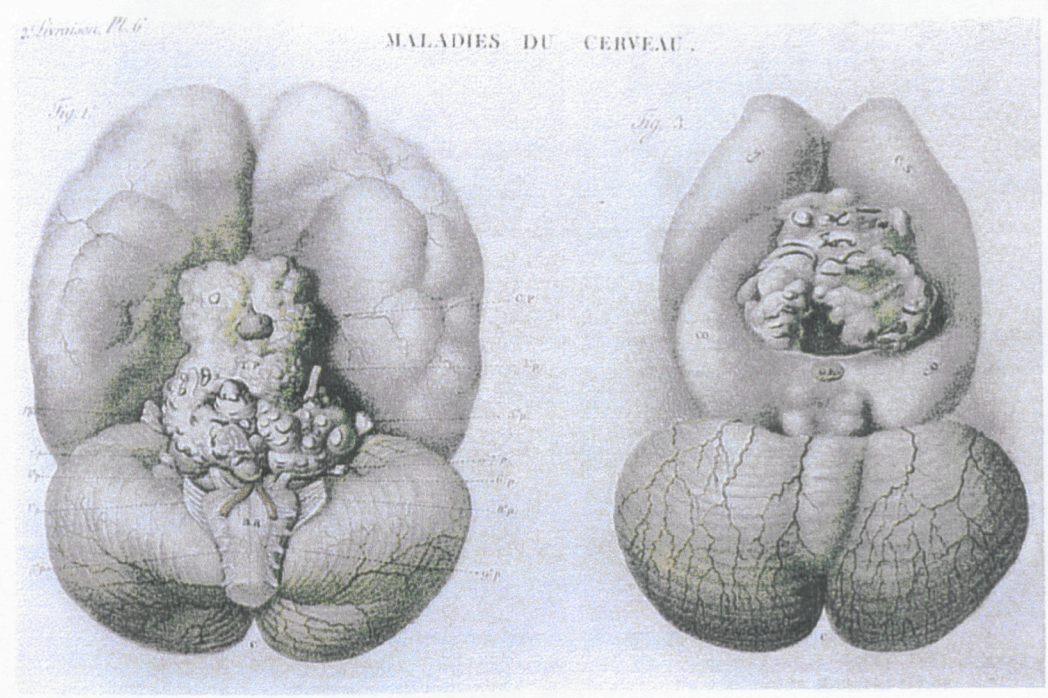
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Cholesteatoma can be described as an accumulation of keratinizing squamous epithelium shed by a proliferating basal layer of skin and which is abnormally located within the middle ear cleft. The skin that generates this mass of shed epithelium originates from the tympanic membrane or ear canal in nearly all cases. There are two major forms of cholesteatoma, namely acquired and congenital with the acquired form being much more common than the congenital type. There are examples of “cholesteatomas” occurring outside the middle ear cleft and these are probably what were described by the early clinicians such as Muller (1838) and Cruveilhier (1829). These forms of “cholesteatoma” do not appear to arise in the same way as the typical acquired middle ear cholesteatoma. They are more likely to be epidermoid (skin) inclusion cysts which are either developmental, as seems likely in Cruveilhier’s case (Fig 1.2.1), or traumatic with viable skin becoming buried beneath the surface.

The general appearance of a typical cholesteatoma is that of a lobulated mass of creamy white, rather greasy tissue enclosed by a thin layer of vascular skin (Fig 1.2.2). The shape of a cholesteatoma arises as it conforms to the surrounding bony walls of the middle ear or mastoid (Figs 1.2.3 & 1.2.4).

In 1974, Schuknecht proposed that if this stratified keratinising squamous epithelium which originated from the external auditory canal or tympanic membrane, invaded and accumulated in the middle ear or other pneumatized areas of the temporal bone, then it can more accurately be described as keratoma. This term has not been generally accepted.

FIG 1.2.1



'Cholesteatoma'

(Pinson's illustrations) First described and presented by Cruveilhier in 1829.



FIG 1.2.2



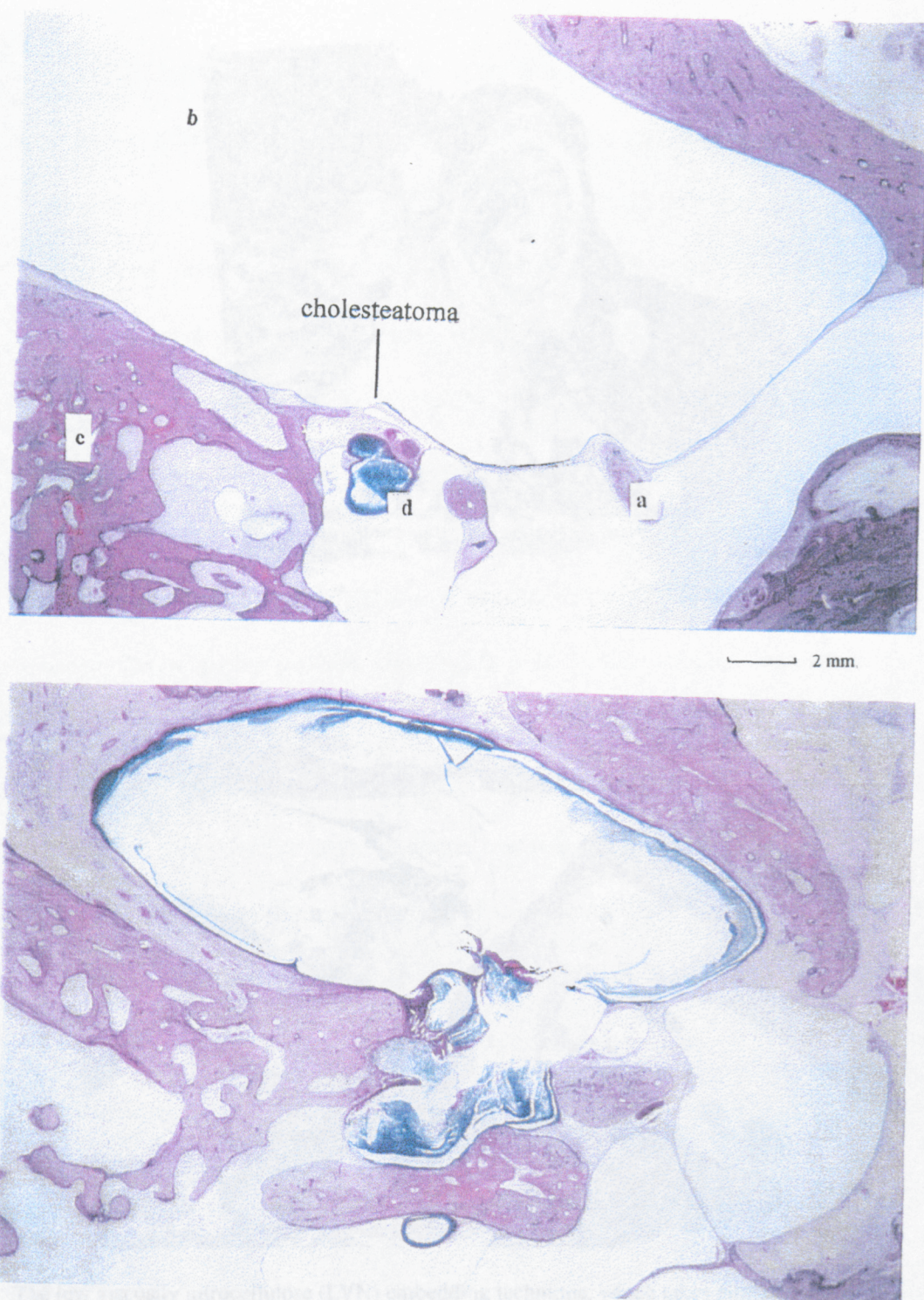
Cholesteatoma : a hollow sphere having an irregular surface with a whitish mother-of-pearl sheen  
diameter: 1.5cm



On dissection, a soft waxy cream onion skin, sometimes papillary and containing keratinised epithelium.



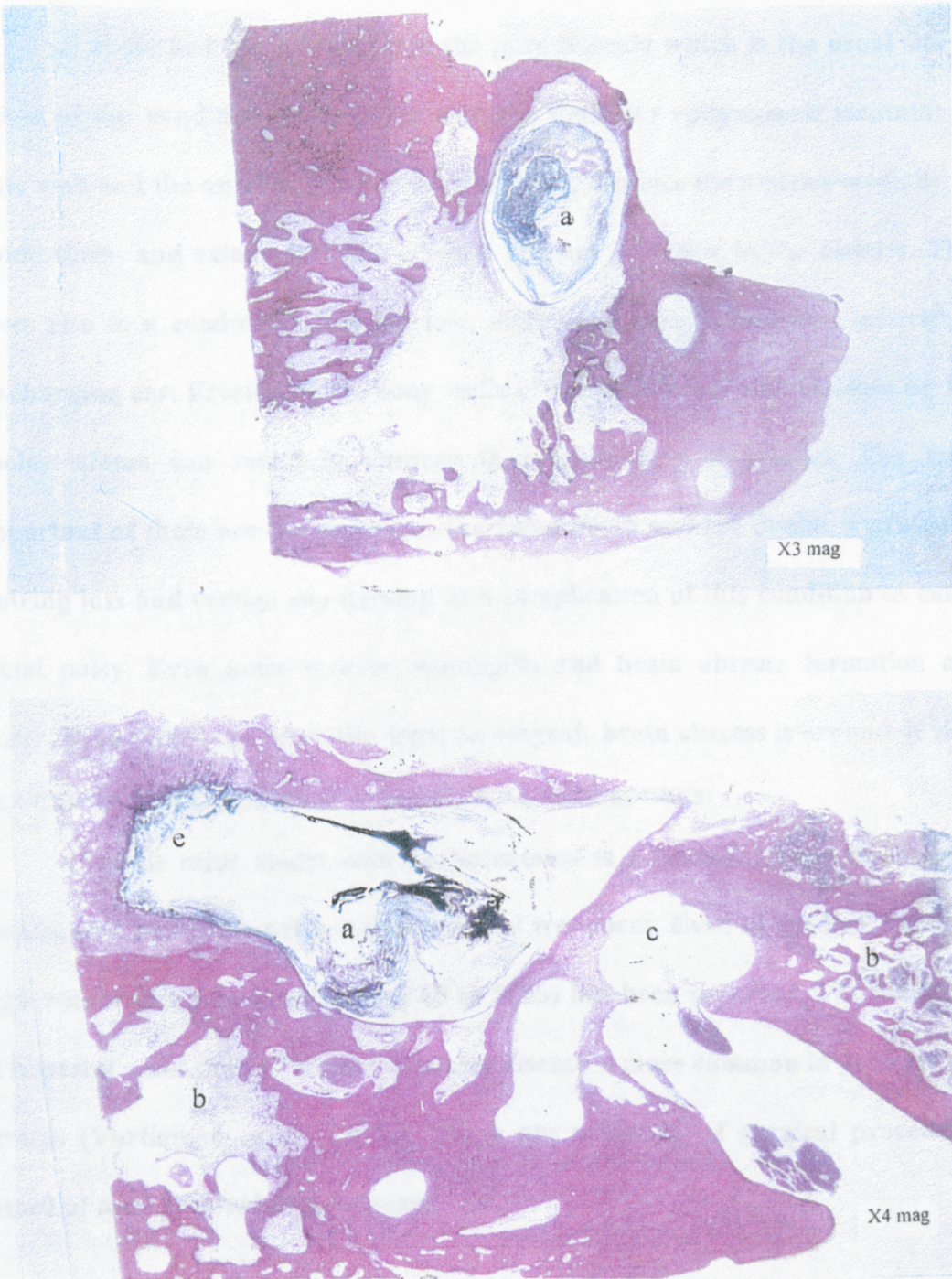
FIG 1.2.3



Temporal bone sections processed, cut and stained by Tony Frohlich (a former colleague) illustrate a cholesteatoma which was undetected prior to death. Only the tip of the cyst could be seen in the first section. Further, serial sections reveal its extent, lining the middle ear cavity. Other features include a) malleus, b) external ear canal, c) mastoid bone and d) trapped keratin squames.



FIG 1.2.4



The low viscosity nitrocellulose (LVN) embedding technique, which takes nine months to complete, is paramount for the preservation in sections of a) cholesteatoma epithelium and keratin squames, b) mastoid bone cavities, c) cochlea and e) the evidence of bone erosion.

### **1.2.3 CHOLESTEATOMA: LOCATION IN VIVO**

A cholesteatoma arising from the pars flaccida which is the usual site of origin of this condition, initially lies between the outer epitympanic (scutum) or attic wall and the ossicles. It can erode this wall, displace the ossicles medially or erode them, and extend into the mastoid antrum posterior to the ossicles. This gives rise to a conductive hearing loss, and if the keratin becomes infected, a discharging ear. Erosion of the bony walls of the middle ear and mastoid by the cholesteatoma can result in damage to neighbouring structures. The most important of these are the inner ear, the facial nerve and the brain. A profound hearing loss and vertigo can develop as a complication of this condition as can a facial palsy. Even more serious, meningitis and brain abscess formation can occur. Even today, the mortality from an otogenic brain abscess is around 40%.

### **1.2.4 CLINICAL MANAGEMENT AND SURGICAL PROCEDURES**

Chronic otitis media with cholesteatoma is a potentially life-threatening disease and nearly always requires surgical treatment. Even in the best hands, a high recurrence rate after surgery (5 to 50%) has been reported. The incidence of bilateral cases (8 to 17%) and bilateral disease is more common in younger age groups (Vartiainen *et al.*, 1993a). There are a variety of surgical procedures aimed at total removal of the disease.

### **VENTILATION TUBES**

Invagination of the tympanic membrane caused by a reduced gas pressure in the middle ear cavity and resulting from dysfunction of the Eustachian tube is



negated when the tympanic membrane is perforated (Honda 1989) and while this supports the use of ventilation tubes Diamant (1981), other researchers claim that reduced pressure does not play a significant role in bringing about the formation of cholesteatomas (Austin (1977). Histological evidence of differentiation between retraction pockets and cholesteatoma can be difficult as rete pegs and papillae are only found near perforations and close to the spur shaped advancing edge of a cholesteatoma. In front of this the stroma is generally bare and an exposed connective area thus separates the middle ear epithelial lining from the intrusive epidermis (Gratacap *et al.*, 1993). Normally, there is no direct contact between epithelium and bone tissue.

Complications following the natural extrusion of ventilation tubes, previously reported in 0.6 to 3.2% of cases between 6 weeks and 5 years is now as high as 10.3% in children 2-13 years (Solomon 1993), lasts longer than six months and is unlikely to heal. It is therefore recommended that edges of perforations of tubes which need removal rather than await spontaneous extrusion, be freshened until bleeding before applying a gelfoam patch to promote healing. Postoperative cholesteatoma develops in 0.7% primary myringoplasty operations (Vartiainen *et al.*, 1993a). Repair involves granulation tissue and the presence of prostaglandin E<sub>2</sub> which normally inhibits epidermal proliferation but, combined with endotoxin stimulates epidermal proliferation *in vitro* (Sugita *et al.*, 1986) and, in addition, the presence of fibronectin also induces epithelial migration during wound healing.

#### 1.2.5 THEORIES OF CHOLESTEATOMA FORMATION

In the absence of a proven simple root cause of cholesteatoma, consideration has been given to a multiplicity of origins by many authors. Over the past century, theoretical propositions which lack proof are frequently discarded, only to be resurrected and repropounded.

For example:

Metaplasia of the epitympanic mucosa (Ulrich 1917, Sade 1977).

Implantation from surgery (Vartiainen et al., 1993b).

Atelectatic retraction pockets (Bezold 1890, 1908).

Migration and marginal perforation, based upon animal models (Habermann 1889, Lange 1925, McKenzie 1931, Ruedi 1959, Fernandez *et al.*, 1960, Vassali 1988, Masaki *et al.*, 1989, Wright *et al.*, 1991, Chole *et al.*, 1993, Imamura 1993).

Proliferation of the germinative layer of Shrapnell's membrane (Lange 1925).

Spontaneous occurrence (Chole 1982, Fulghum *et al.*, 1985).

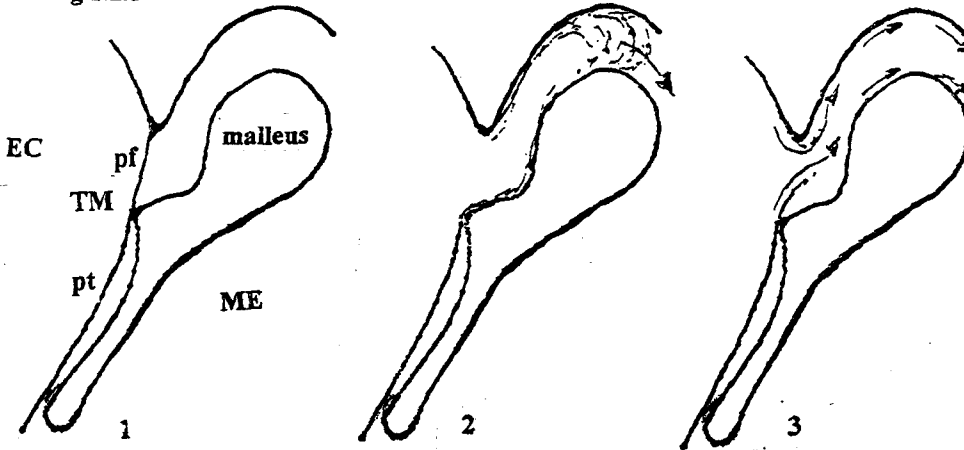
Some of these theories will be discussed later.

In general the acquired cholesteatoma theories fall into two major groups namely epithelial ingrowth or development from retraction pockets.

#### 1. BY WAY OF PERFORATION OF THE TYMPANIC MEMBRANE (Fig 1.2.5)

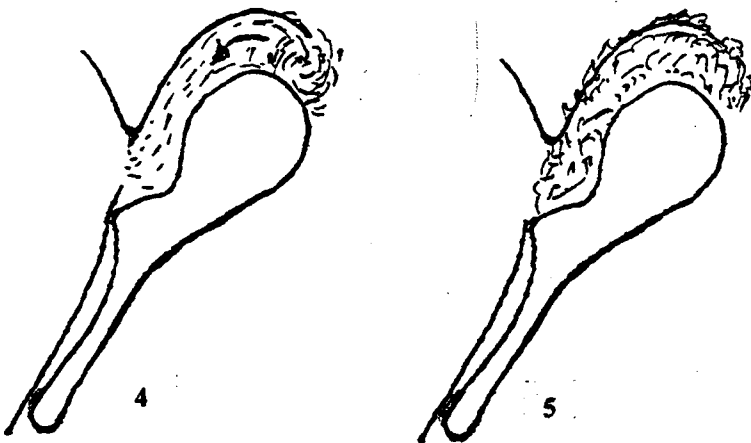
Following the invasion of migrating epithelium around the edge of a tympanic membrane, perforations which probably occur more commonly in the pars flaccida than the pars tensa, an accumulation of keratin can result and form a cholesteatoma. The cholesteatoma can extend posteriorly into the antrum, periantral cells and central mastoid tract or inferiorly, into the middle ear.

Fig 1.2.5 ORIGINS OF CHOLESTEATOMA



key EC: External Ear Canal. ME: Middle Ear Cavity  
TM: Tympanic Membrane, pf : pars flaccida pt: pars tensa

(fig 1) Normal. (fig 2) Retraction Pocket: compared with atmospheric pressure in the external ear canal, continued negative pressure associated with otitis media with effusion in the middle ear can produce a negative retraction pocket in the attic which, because of its narrow neck, becomes a cholesteatoma. (fig 3) Attic perforation, caused by acute otitis media, enables squamous epithelium to grow in from the external auditory meatus.



Metaplasia: subsequent to otitis media, the mucosa undergoes metaplasia to squamous epithelium which either (4) forms into a cyst which eventually bursts externally as a cholesteatoma or (5) causes epithelium overlying the attic to hypertrophy and form a cholesteatoma.

Posterosuperior perforation of the pars tensa leads to cholesteatoma of the posterior mesotympanum and extending to the epitympanum and the mastoid.

## **2. RETRACTION POCKETS (Fig 1.2.5)**

Obstruction of the Eustachian tube, followed by the presence of oedema and granulation tissue may all contribute to a sustained or fluctuating negative middle ear pressure and result in a flaccid tympanic membrane. Such membranes are prone to retraction, especially in the posterior marginal and attic regions which usually form the perforations or openings and, as they enlarge, lead to cholesteatoma. Even so, cholesteatoma formation is rare in such circumstances compared with the incidence of chronic secretory otitis media.

## **3. METAPLASIA OF MIDDLE EAR MUCOSA (Fig 1.2.5)**

It has been suggested that the respiratory mucosa of the middle ear undergoes metaplasia into squamous epithelium which subsequently proliferates and keratinizes to form a cholesteatoma in the middle ear (Michaels 1982, Sade *et al.*, 1983).

Squamous metaplasia of the inflamed ear with keratin production found much support (Paparella 1964, Palva *et al.*, 1968, Sade 1983 and Hentzer 1972). Examples of metaplasia include transformation of respiratory epithelium to epidermoid squamous epithelium in the bronchus with identification of basal, neurosecretory, mucous, ciliated and undifferentiated cells. Reverse transition of squamous epithelium to respiratory, by increasing carbon dioxide concentration on nasopharyngeal mucosa (Sade 1979) and by the depletion of vitamin A as seen in laryngeal mucosa was also noted, suggesting that the mechanism could exist (Young 1971). Small numbers of vacuolated cells in

stained sections of cholesteatoma from the middle ear were presented as an explanation of the origin of cholesteatomatous squamous epithelium directly from the mucous secreting epithelium in the middle ear (Michaels 1982). A further observation (Bujia *et al.*, 1993a) that the vast majority of cells infiltrating the stroma consists of T-cells and macrophages suggests these may be responsible for keratinocyte dysregulation in cholesteatoma epithelium.

Those not in favour of a metaplastic origin cite robust immunohistochemical findings (Boedts *et al.*, 1982, Lepercque *et.al.*, 1993) that acquired cholesteatoma lacks evidence of any of the major and typical cytokeratin constituents of the middle ear mucosa.

It seems extremely unlikely that metaplasia of middle ear respiratory mucosa into squamous epithelium could account for cholesteatoma formation, especially as tissue culture techniques have not produced squamous metaplasia that is migratory.

#### 1.2.6 ANIMAL MODELS (Fig 1.2.6)

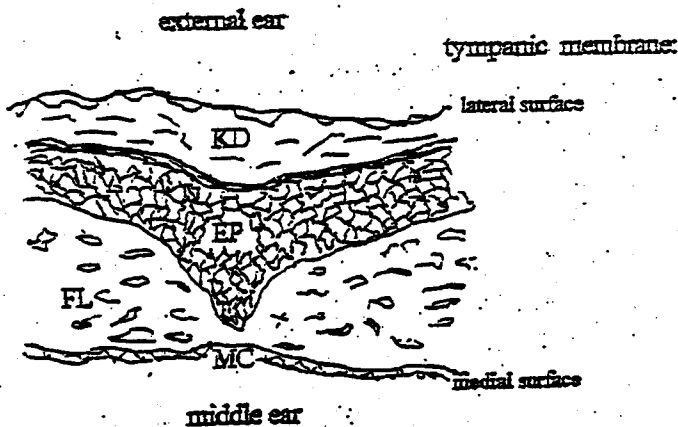
Using animal models, the formation of cholesteatoma can be achieved by introducing a variety of substances into the middle ear. The animal models include guinea pigs, rabbits, gerbils, chinchillas and rats and the agents used to induce cholesteatoma include ethylene glycol (Vassalli *et al.*, 1988b), talc, fibrin and gelfoam (Steinbach *et al.*, 1980). These can destroy the mucosa lining the middle ear and the inner surface of the tympanic membrane within forty eight hours. The fibrous layer of the TM is usually left intact. Re-epithelialization takes place within about four days. However, if the damage is more severe and defects to the medial surface of the fibrous layer occur, papillary projections of

cones to penetrate these defects and can produce a cholesteatoma (Chole 1985). A carcinogen, dimethylbenzanthracene injected into the middle ear produces typical cholesteatoma with bone destruction and formation (Schmidt *et al.*, 1993).

Ruedi (1978) demonstrated papillary ingrowth through an intact animal membrane (see Animal Models) and proposed that this route may occur in man, although this has received little further support. It is a common clinical observation that once formed, small retraction pockets of the pars flaccida start to accumulate keratin which subsequently becomes trapped and forces the pocket to expand as more keratin accumulates.

Fig 1.2.6

Experimental cholesteatoma in the chinchilla following propylene glycol inflammation\* and, sporadically, other chemical irritants\*\*.



KD: keratinaceous debris, EP: hyperplastic epidermis penetrating FL: fibrous layer and MC: mucosal layer prior to proliferating in the middle ear cavity. Vassali *et al.* 1988b, Meyerhoff *et al.* (1990), Wright *et al.* (1991), Lange (1925), Ruedi (1959), Fernandez (1960).

In Mongolian gerbils (Chole *et al.*, 1993), Eustachian tube blockage leads to tympanic membrane retraction and pars flaccida retraction pocket cholesteatomas. This occurs because of the failure of ventilation of the middle ear with the subsequent formation of a reduced middle ear pressure and results in retraction of the ear drum because of pressure difference between the ear canal and middle ear. Any cause of a reduced middle ear pressure can result in a retracted pars flaccida, which can accumulate shed keratin and subsequently develop into a cholesteatoma. Cholesteatoma frequently conforms to the architecture of the middle ear.

TABLE 2. A COMPARISON BETWEEN THE STRUCTURED LAYERS OF TYMPANIC EPITHELIUM  
(see Table 1) AND CHOLESTEATOMA :

<i>Epithelial Layers:</i>	Pars tensa	Pars Flaccida	Cholesteatoma
<i>Stratun corneum</i>	Several layers	Several layers	Multiple layers
<i>S.granulosum</i>	1 - 3 layers	Up to ten layers	Up to ten layers
<i>S.spinosum</i>	4 - 5 layers	4 - 5 layers	4 - 5 layers
<i>S.basal</i>	single layer	single layer	single layer
<i>Fibroblast and Collageous core:</i>			
<i>Connective tissue</i>	Absent	Thin layer	Thin layer
<i>Lamina propia</i>	————— connective tissue with capillaries —————		
<i>Radial fibres</i>	Present	Random orientation	Absent
<i>Circular fibres</i>	Present	Random orientation	Absent

#### 1.2.6 A RECENT APPROACH TO ACQUIRED CHOLESTEATOMA

In a recent approach to the close association between *chronic suppurative otitis media* (CSOM) and *acquired cholesteatoma*, Ludman and Wright, in their *Diseases of the Ear* (1998), regard these two terminologies as being practically synonymous. CSOM arising from the marginal region of the posterosuperior tensa or pars flaccida almost always includes the presence of *cholesteatoma*, terminology which is more apt than the previously termed 'unsafe ears', as opposed to the 'safe', those *mucosal* where infected pus can discharge through a perforation in the pars tensa.

CSOM accompanied by cholesteatoma can be classified as:

- (1) "primary" where there is no previous history of infection, or
- (2) "secondary" which follows or accompanies active infection.

Ludman and Wright further define three types of cholesteatoma which include those occurring:

- (1) via a retraction pocket or perforation of the same, in the postero-superior quadrant of the pars tensa. These, are *pars tensa* types of cholesteatoma, and can be responsible for destruction of the ossicular chain and for producing conductive deafness besides granulations and osteitis in the deep meatus and annulus region.
- (2) *pars flaccida* cholesteatomas, occurring in retraction pockets or perforations in the pars flaccida region of the tympanic membrane and which give rise to osteitis and destruction of the outer attic wall.
- (3) *occult* cholesteatomas where there is an intact tympanic membrane and these cholesteatomas are often congenital in origin.



### 1.2.7 SUPPURATIVE OTITIS MEDIA

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#### THE BACTERIA

*Streptococcus pyogenes* is a common cause of acute suppurative otitis media (personal observation). Misleading findings about its frequency arise in the absence of selective media and indiscriminate or blind treatment using penicillin. *Haemophilus influenzae* is also a common primary pathogen.

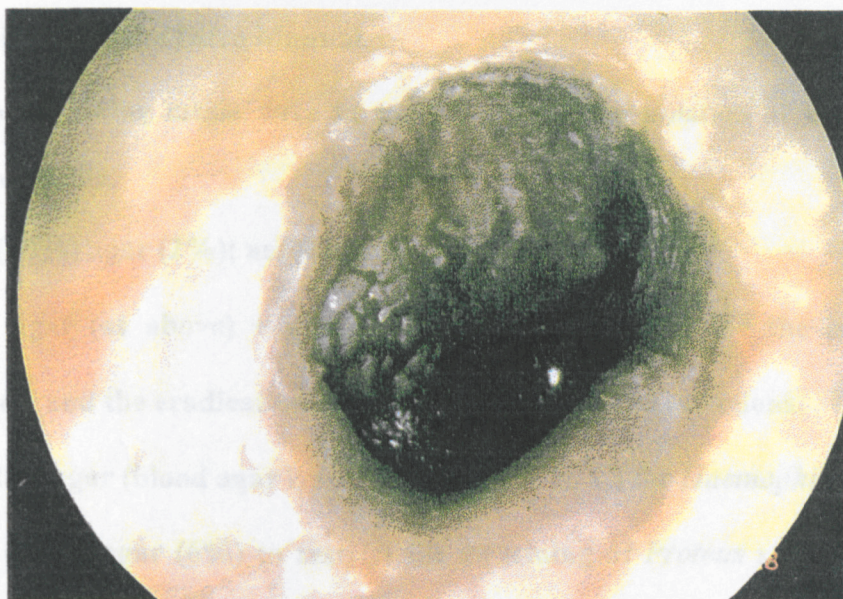
A secondary invader, *Staphylococcus aureus*, is generally regarded as less pathogenic, unless it is methicillin resistant. It produces a proteolytic dermo-necrotic enzyme which, due to the constriction of small veins and capillaries, can adversely affect the pars flaccida.

Invasion by *Pseudomonas aeruginosa* during the chronic phase of infection usually masks the presence of *Streptococcus pyogenes* and destroys *Staphylococcus aureus*, against which it is markedly antagonistic. This scenario again reinforces the necessity of using selective media, listed in the Methods Chapter.

Whereas the potentially injurious effect of many ubiquitous bacteria is well documented, the pathogenicity of *Pseudomonas aeruginosa* (profile in the Appendices) is frequently overlooked. This is hardly surprising in view of reports by Emerich *et al.*, (1902) '*Pseudomonas* are highly bactericidal to many organisms ... but less toxic to tissue cells, Emerich Stitt *et al.*., (1943) reported that 'it can be the sole cause of otitis media and is more pathogenic than generally

supposed' and Dubos (1948) 'its pathogenicity is of low order .... causing suppurative lesions in otitis media'.

FIG 1.2.7



**This shows an acute infection of the middle ear. The posterior portion of the membrane is red and bulging because of the presence of acute inflammatory fluids and probably pus in the middle ear.**

My findings in bacteriology, over a twenty year period (1960-1980), at the Royal National Throat, Nose and Ear Hospital (RNTNE), London were based upon formulae and methods in contemporary editions of Mackie and McCartney's Handbook of Practical Bacteriology, Topley and Wilson's Principles of Bacteriology, revised by Wilson and Miles, Cruickshank's Medical Microbiology and the Difco Manual.

The following media were in routine use for the isolation of bacteria from suppurative media.

1. Blood (10%) agar (2%); aerobic and anaerobic incubation.
2. Blood agar (as above) + gentian violet (1 in 500,000) for the isolation of *Streptococci* and the eradication of *Staphylococci* in mixed infections.
3. Chocolate agar (blood agar lysed by heating at 60°C) for *Haemophilus* species.
4. Blood (10%) agar (6%) to restrict the swarming of *Proteus* species in mixed infections and allow for the isolation of other species of bacteria.
5. Mc Conkey's bile-salt, neutral red lactose agar for the isolation and primary identification of lactose fermenting Gram-negative bacilli (e.g., *Escherichia coli*).
6. Liquid Robertson's cooked meat medium for the primary culture of anaerobic bacteria and bacteria present in scanty numbers. This broth was subcultured following 24h and 3d incubation.

Additional media consisted of Lowenstein Jensen and Dorset's Egg used for Mycobacteria infections, Sabouraud agar for yeasts and fungi. Stuart's transport medium was used for specimens subjected to delay during delivery to the laboratory.

Cholesteatoma is a pathological, proliferating mass of stratified keratinising squamous epithelium which has become trapped within the confines of the middle ear and mastoid cavity. In other respects it is similar, if not identical, with regenerating epithelium migrating across the healthy external surface of the normal ear drum. Cholesteatomatous epithelium, however, continues to migrate and proliferate, accumulating dead squamous epithelial cells (squames) in the middle ear and mastoid and producing chronic inflammation. Complications can involve damage to the Facial (VII) nerve causing facial palsy; damage to the ossicular chain causing a conductive deafness and loss of hair cells to the inner ear, causing profound deafness and vertigo. The need for surgical intervention may arise because of the potential danger of the condition, but surgery is itself not without risk to the individual.

The factors associated with the intitial origin and formation of cholesteatomas are not well established and could be relevant in the prevention of this disease. Comments from clinicians about the incidence in cholesteatoma of chronic ear infection and in perforations of the tympanic membrane and a review of the literature suggest that infection may be important in the initial development of a cholesteatoma. This thesis then

attempts to provide a partial explanation of some of the major initiating factors involved in this important condition and explores in three experimental approaches, some of the factors that may be involved in the first stages of cholesteatoma formation and growth, namely:

(1) The morphology and migration pattern of the normal tympanic membrane epithelium.

(2) The morphology of cholesteatomatous epithelium during en masse migration.

## CHAPTER 2: METHODS

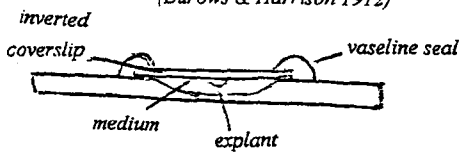
### 2.1.1 TISSUE CULTURE: HISTORICAL

Explantation from animals during the last century probably marked the primitive beginnings of tissue culture when the medullary plate of a chick embryo was maintained in saline for several days prior to transplantation into another animal of the same species (Roux 1885). The grafting of human skin which had been stored in ascitic fluid proved to be successful and Jolly (1903), was able to maintain salamander leucocytes *in vitro* and observed mitoses indicating that the cells were not just surviving but were also actively growing.

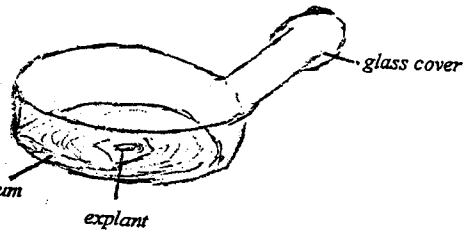
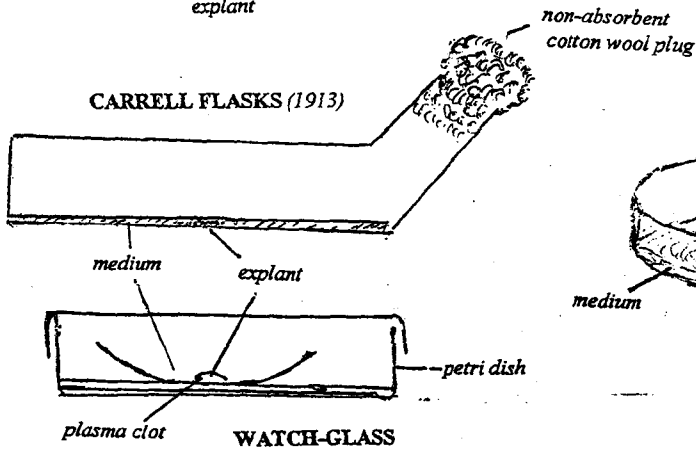
The potential advantages of culturing tissue, *in vitro*, was fully realised when fragments of tissue dissected from the medullary region of frog embryos and cultured in frog lymph produced an outgrowth of nerve fibres (Harrison 1907). Burrows (1912) introduced the well slide on which a coverslip culture could be inverted and sealed with a ring of vaseline to form a simple culture chamber (Fig 2.1.1). Carrell (1913) propagated a strain of animal cells over several years, by growing tissue in plasma clots supplemented with embryo extract. The introduction of the 'Carrell' flask design, with a convenient short and angled neck, allowed a pipette to be inserted easily to the medium to replenish it. With the advent of disposable plastic replacing recycled glass many of these earlier innovations remain in use at the present time. Many of our abbreviations e.g., 'DMEM' (*Dulbecco's* modification of *Eagles* Medium) remind us of some pioneers in tissue culture and bursaries register the names of scientists working with tissue culture e.g., Honor Fell who was responsible for the beginnings of organ culture.

# 'WELL' SLIDE

(Burows & Harrison 1912)



## CARRELL FLASKS (1913)



## Tissue Culture Flask with Bacteria filter cap

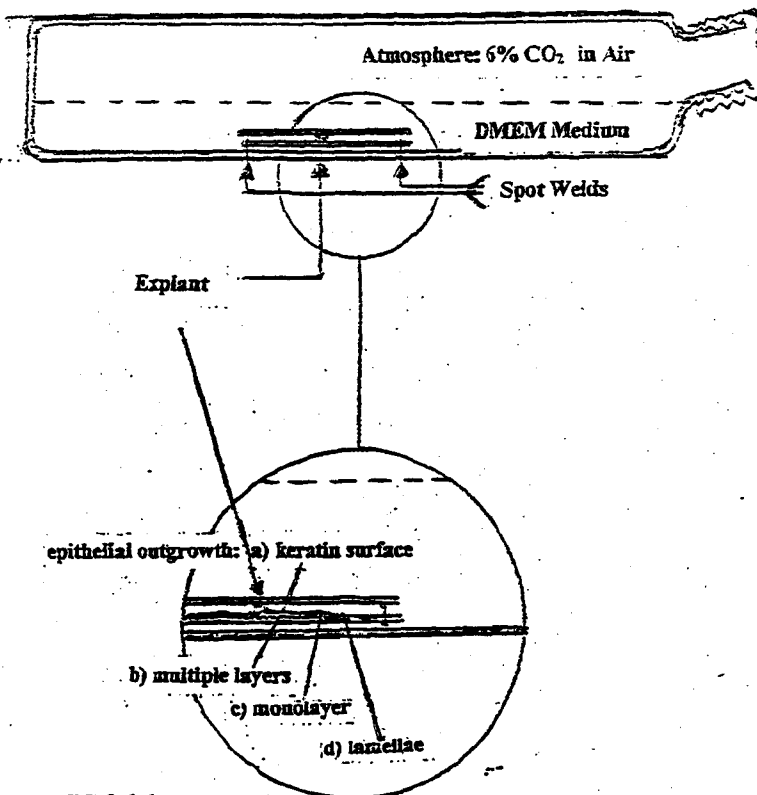


FIG 2.1.1

Sandwich coverslip preparation.

The first tissue culture experiments at the Institute of Laryngology and Otology (ILO) were carried out as part of the histopathology section by Ernest Bird under the direction of Professor I. Friedmann, Head of the Department of Pathology and Bacteriology. 53 experiments were recorded on ciliary movement in nasal mucosa using embryonic otocysts (ILO Annual Reports 1954).

During the 1960's, following the introduction of established human cell cultures which could be propagated indefinitely (e.g., HeLa), a separate tissue culture and virology section was founded at the ILO in response to Professor Friedmann's intentions to increase research initiative. The aspirations were twofold: as research, to compare cultures of normal and neoplastic epithelium and, as service work, to establish a diagnostic virology laboratory based on tissue culture. The successful demonstration of intracellular Adenovirus using transmission electron microscopy, a new tool at the ILO, was impressive but this approach and making use of the cytopathic effect (CPE) of viruses on tissue cultures proved too slow and unreliable as a useful aid to clinical diagnosis and CPE was soon to be superseded by fast, reliable, diagnostic serology.

In common with other research workers, no satisfactory method was achieved for the culture and propagation of normal and neoplastic epithelium tissue extending to more than a few days duration. The addition of growth factors was in its infancy and what at first appeared to be promising cultures of epithelium were either overgrown by fibroblasts, whose rate of growth



exceeds that of epithelium, or the epithelium perished through lack of nourishment from epithelial growth factor.

The breakthrough in maintaining successful prolonged cultures of epithelium, without fibroblast overgrowth, finally arrived during 1975 with Rheinwald and Green's feeder layer of *irradiated* fibroblasts which continued to secrete growth factors without proliferating. Their method, combined with the careful testing and selection of fetal calf serum specifically screened for its ability to grow keratinocytes, facilitated a study of the differences between squamous cell epithelium of the human larynx and control epithelium taken from human prepuces (Boxall *et al.*,1986). The use of additional growth factors, epidermal growth factor (Rheinwald and Green 1977), cholera toxin (Green 1978) and hormones (Rheinwald and Green 1980), proved to be useful adjuncts to calf serum.

### 2.1.2 CURRENT TISSUE CULTURE METHODS

---

Two basic schools of approach have developed in the tissue culture of epithelium: (1) the simple culture of epidermal explants and (2) the culture of epidermal cell suspensions obtained by enzyme dissociation of epidermis or epithelium outgrowths from explants. I used both methods for the culture of *tympanic membrane epithelium* (TME) and *cholesteatomatous epithelium* (CHE) for the following reasons.

On the one hand, an explant, at least initially, is representative of the original tissue. It is accompanied by other cells including macrophages, dendritic cells, lymphocytes and epidermal growth factors with the exception of glutamine which is thermolabile above -20 °C. These natural nutriment avoid the need to quantify and set up individual controls against unnatural or volatile additives. Explant outgrowths of TME cultured on a suitable substratum, migrate *en masse* away from the explant, after some 10 days incubation, in a linear direction. Even scanty outgrowths, emanating from less than 1% of the circumference of an explant, can survive. In addition, some cholesteatoma explants yield abundant epithelium even though adjacent explants, which appear to be identical, yield none.

On the other hand, and providing that the harvest or yield from TME dissociated by enzyme treatment is sufficiently viable for proliferation, then the migration pattern of established epithelial colonies, without the barrier presented by an explant, is different and warrants separate study.

## **(1) SIMPLE EXPLANT TECHNIQUE**

During earlier work (Boxall *et al.*,1988) explants were placed on coverslips for one hour, before covering them with additional coverslips. This standard technique was intended to allow explants to semi-dry and anchor themselves without adverse effects. However, the subsequent use of serum-free medium often destroyed cell attachment and fibrin or collagen was needed to supplement this loss. Therefore, I devised a simple instant sandwich technique (Fig 2.1.1) which avoided this problem besides allowing for video time-lapse recordings without disturbing the explant. In addition, the undisturbed explant and epithelial outgrowth could be removed from a culture flask and the coverslips gently prised apart for subsequent fixation, staining and electron microscopy. This successful technique continued in routine use, as follows:

During gross dissection, any floating scales of dead epithelium were discarded. Fragments to be used as explants ( $<1\text{mm}^3$ ) were placed on rectangular tissue culture coverslips 22 X 5 X 0.15-0.185 mm and an additional coverslip was placed over each mounted fragment to form a sandwich. The two coverslips were placed in disposable plastic tissue culture flasks and loosely spot-welded together, using the flame of a Bunsen burner to heat the tip of a needle to red heat which was then applied to each end of the coverslips and to the bottom of the plastic culture flask.

The growth of the epithelium was unimpeded and, frequently, both faces of the opposing coverslips were populated with epithelium. This

depended upon successful orientation of the basal layer of epithelium being placed against the coverslip. Unlike propagating epithelium which remained captive on the coverslips because their lamellae are on the apical surface of the leading cells (Boxall *et al.*, 1990) and proboscis upturned, the lamellae of the fibroblasts enabled them to migrate across the edge of the coverslips and populate the flasks.

The average life-span of the en masse migrating epithelium outgrowth using this method and feeding the cultures at four day intervals, without subculture, was about two months.

One of the most important improvements during the past few years has been a major advance in the reliable quality of commercial fetal calf serum. At least one supplier (Sigma Ltd., UK) supplies free serum samples for testing purposes and offers to retain sufficient stock of a selected batch, until it is required, at no additional cost. Sigma's low temperature storage facility avoids deterioration up to 4 years, enabling most investigations to be completed besides avoiding the risk of breakdown in the laboratory deep-freeze refrigerator.

The rationale behind using this very simple explant regime was to compare cultures of natural cholesteatoma epithelium in the presence, at least initially, of accompanying cell populations and to avoid cloning or using immortalized cells. A further reason was to restrict changes brought about by additives unless they were essential for the maintenance of healthy growth, i.e., to restrict the medium to a buffered salt solution, carefully selected fetal calf serum (FCS) and glutamine. The major cause of failure during the 1960's was

the poor quality of FCS; failure to test a large number of batches prior to selection and the safety of bulk storage at a very low temperature. Serum-free medium has not proved consistently successful and, again, requires organic supplements.

Evaporation of moisture during the preparation of culture explants can produce an increase in the viscosity of the plasma present and, together with the presence of red cells, effectively impede the outgrowth of epithelium, whilst allowing the survival and propagation of fibroblasts (Fig 2.1.2).

Cholesterol crystals, oblong in shape with notched corners are frequently present (Sugita *et al.*, 1989) and their presence is noted although their significance, at present, remains uncertain.

## **(2) KERATINOCYTE CULTURE USING ENZYME DIGESTION**

Dissociation of epithelium can prove difficult because of the tight bonds between the cells. The effect of the enzyme trypsin is enhanced by the addition of a chelating agent, ethylenediaminetetraacetic acid (EDTA) in calcium and magnesium-free phosphate buffered saline (PBS), which depletes the calcium in desmosomes.

Sterile, tissue culture grade disposable plastic was used throughout the procedures.

The epidermal tissue for culture was placed in a Petri dish containing 10 ml Dulbeccos Modification of Eagles Medium (DMEM) . Any connective tissue was dissected away and discarded. Alternatively, the epidermis was placed in 20ml dispase, (1000 protease units/ml) at 4°C, overnight which rendered the dermis easier to separate. The epithelium was cut into small

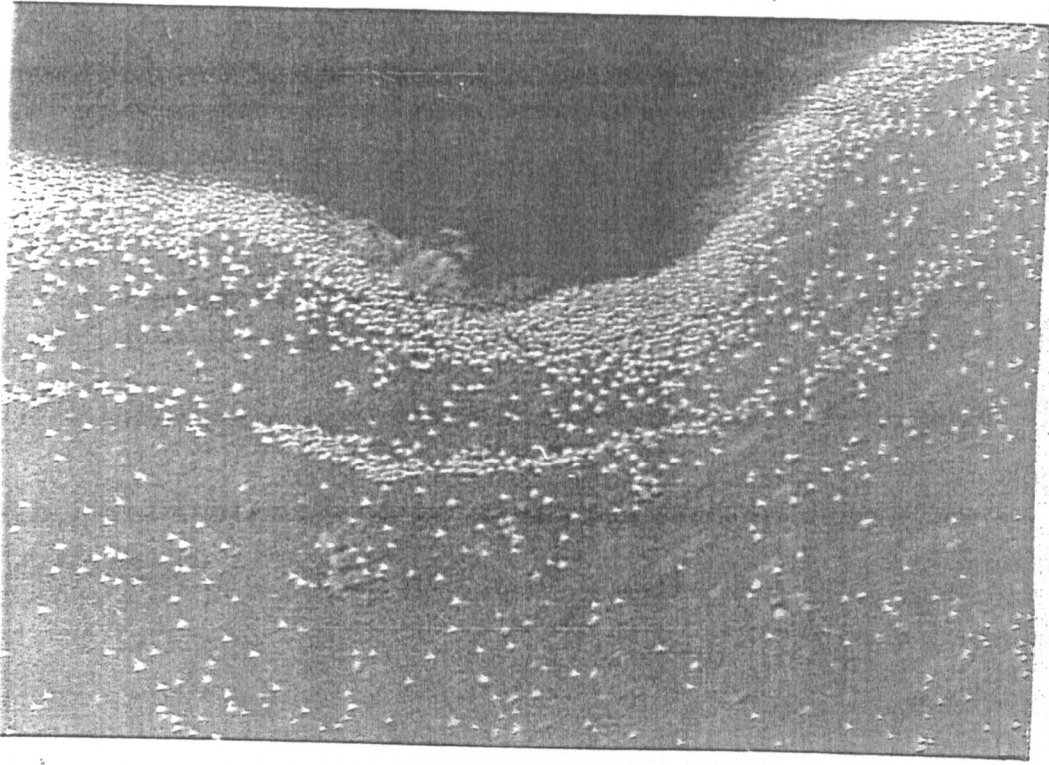
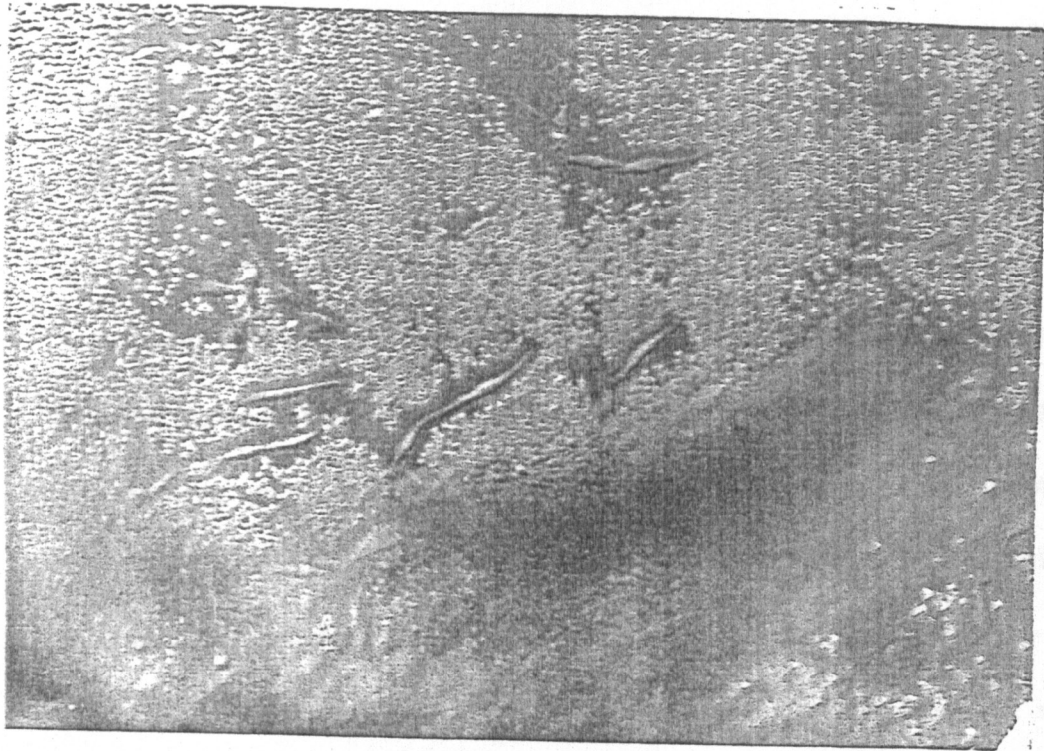


FIG 2.1.2 The explant occupies the top portion of the photograph and a line of red cells shows the demarcation where serous exudate has been allowed to partially dry, before the addition of culture medium. — 100  $\mu$ m



The presence of fibroblasts *in situ* at the time of mounting an explant and unaffected by erythrocytes

fragments ( $<0.5\text{mm}^3$ ) and transferred twice to fresh DMEM before being placed in a mixture of 10 ml trypsin (0.25%) + 10 ml EDTA (0.025%) in phosphate buffered saline at  $37^\circ\text{C}$  and left until the cells were seen to be dissociated after gentle shaking. 2ml of soybean (0.1%) in buffer was added as an inhibitor, the suspension of cells lightly centrifuged ( $<1500\text{ rpm}/5\text{min}$ ) and resuspended in growth medium consisting of DMEM containing 10-20% Fetal Calf Serum\* (FCS) selected to grow keratinocytes; Penicillin 100 iu/ml Streptomycin  $100\mu\text{g}/\text{ml}$  and Glutamine  $0.4\text{ mg}/\text{ml}$ . The cells were enumerated and seeded at a density of  $4 \times 10^5/5\text{ cm}$  culture dishes or flask, pro-rata. The culture medium was renewed every third or fourth day.

Additives to improve the growth rate, life span and colony forming efficiency included the following: Hydrocortisone  $0.4\mu\text{g}/\text{ml}$  (stock  $4\text{mg}/\text{ml}$  in ethyl alcohol,  $-20^\circ\text{C}$ ), Epidermal Growth Factor (EGF)  $10\text{ ng}/\text{ml}$  (stock  $100\mu\text{g}/\text{ml}$  in DMEM,  $-20^\circ\text{C}$ ), Cholera toxoid  $10^{-10}\text{M}$  (stock  $10^{-5}\text{M}$ ,  $4^\circ\text{C}$ ) and Insulin  $5\mu\text{g}/\text{ml}$  (stock  $5\text{mg}/\text{ml}$  in  $5\text{mM HCl}$ ,  $-20^\circ\text{C}$ ).

Irradiated 3T3 Swiss mouse fibroblasts were used as a feeder layer cultured in DMEM + 10% FCS and subjected to a weekly passage. Fresh cells were thawed every three months or when the cultures showed senescence. Following gentle rinsing in phosphate buffered saline, suspensions were prepared from monolayers using either trypsin/EDTA and reseeding at a dilution of 1 in 10 ( $2 \times 10^5$ ). Keratinocyte cultures were added to the flasks at the same time or within 3 days. Batches of FCS were screened using the control epithelium and those found to be suitable for the culture of keratinocytes, were usually found to be suitable for the culture of fibroblasts.

### 2.1.3 TISSUE CULTURE: SAMPLE COLLECTION AND DISSECTION

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Suitable pieces of human tympanic membrane (TM) and cholesteatoma tissue obtained at otological surgery and from adults up to 12 h post-mortem were placed in transport medium consisting of Dulbecco's modification of Eagle's medium (DMEM) containing penicillin 600 IU/ml and Streptomycin 600 µg/ml and transported immediately to the laboratory. Some samples subjected to unavoidable delay were, however, found to be viable after delays of up to six days, at room temperature (20 °C).

In the laboratory, precautions were used to protect the operator from viral or bacterial infection and the tissue sample from contamination. These precautions included the use of a class 1 safety cabinet, protective clothing, hot air oven and autoclave for sterilising instruments and discards containing Hycolin and bleach. The incubators had a water saturated atmosphere containing 6% carbon dioxide gas.

The gross appearance of the specimen was noted, including colour, approximate size, presence of a typical cholesteatoma cyst or sac, the number, size and consistency of loose fragments and, microscopically, the presence of free squamous cells, red cells, and cholesterol crystals.

A sample of the tissue was cultured in media suitable for the isolation and identification of bacteria (see Media for the Primary Isolation of Bacteria).



The remaining tissue was briefly rinsed in several (6x50ml) changes of fresh transport medium in order to reduce the concentration of any bacteria and excessive numbers of red cells. This brief rinse was intentionally insufficient to remove lymphocytes, macrophages or dendritic cells.

During dissection, careful note was made of the position and orientation of sections and fragments dissected from the whole fetal membranes and the location of epithelial explants taken from cholesteatomas especially in relation to the neck or base of a cholesteatomatous sac.

#### 2.1.4 FOETAL TYMPANIC MEMBRANES

Before restricting legislation had been enacted which restricted this practice, TM's were taken from foetuses aborted between 14 and 19 weeks gestation and much valuable information was gained during tissue culture. An approximate age of a foetus can be obtained by measuring the overall size, e.g., 2nd month: 5-30mm, 3rd month: 31-60mm, 4th month: 61-100 mm, 5th month: 101-150mm, 6th month: 151-200mm (Hamilton, Boyd and Mossman).

The foetal ear drum is an early development, reaching adult size prior to birth. It is located close the surface prior to full development of the external meatus and required only simple dissection by horizontal incision into the infratragal notch, after which the ear canal was widened and the whole

tympanic ring and membrane removed free from surrounding tissue and the underlying primitive mesenchyme.

The membranes were cut into 0.5 - 1 mm squares and cultured using the sandwich technique (see methods). With careful handling their original orientation and position on the drum was recorded and maintained during culture. The culture of whole membranes in order to study migration met with limited success because it proved difficult to facilitate constant contact between fresh culture medium and the whole surface of the membrane.

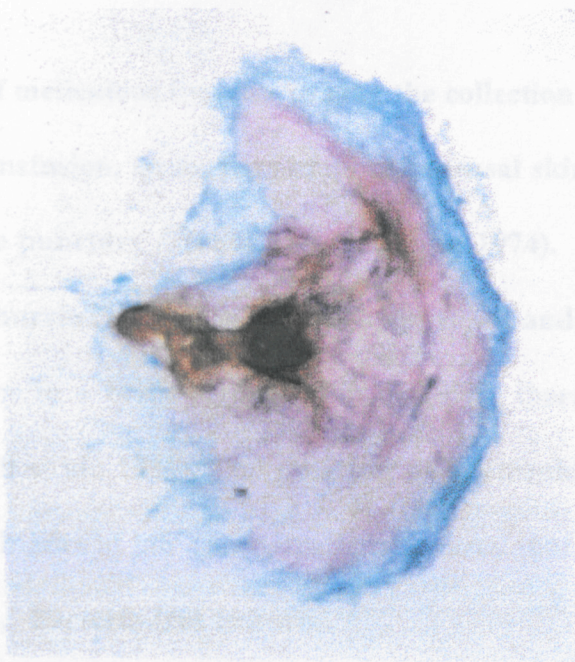
### **2.1.5 TISSUE CULTURE : CONTROL EPITHELIUM**

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In order to make comparisons between the migration of cholesteatoma epithelium, which was available in plentiful supply, and normal tympanic epithelium, which was always in short supply, video recordings of the latter together with fetal and cadaver membranes were used as normal controls.

Initially, infant prepuce epithelium and non-migrating vertically stratifying skin from the extraconchal part of extended endaural incisions, used in ear surgery, were obtained for the purposes checking the suitability of batches of tissue culture media and environment. Fresh skin from my forearm proved consistently viable and being instantly available was used, as the normal non-migrating control epithelium, throughout this thesis (Fig 2.1.3).

In order to collect this, the forearm is thoroughly scrubbed for a few minutes using soap and water to loosen and remove dead skin and bacteria, dried with a sterile towel and the skin surface sterilised by briskly rubbing using iso-propyl alcohol swabs. Avoiding scar tissue and hairs, skin on the inner side of the forearm is squeezed between 12-15 cm straight forceps, allowing a length of skin 1cm x 0.5 - 1 mm to protrude above the shanks. Up to three slices of epidermis 1mm x 5 - 8mm are collected in this manner from three separate areas by slicing the surface using a number 11 scalpel blade, without causing discomfort or excessive bleeding. These epidermal strips are immediately placed in a petri dish containing Dulbecco's Modification of Eagle's Medium (DMEM) and cut into 1 sq mm pieces. With care, inclusion of the dermal layer containing fibroblasts can be avoided.



Subcultures of trypsinized cholesteatomatous epithelium migrate en masse.

Using Ayoub-Schlar's staining method the non-keratinised epithelium stains blue and keratinised epithelium stains red.

(Boxall et.al., 1988)

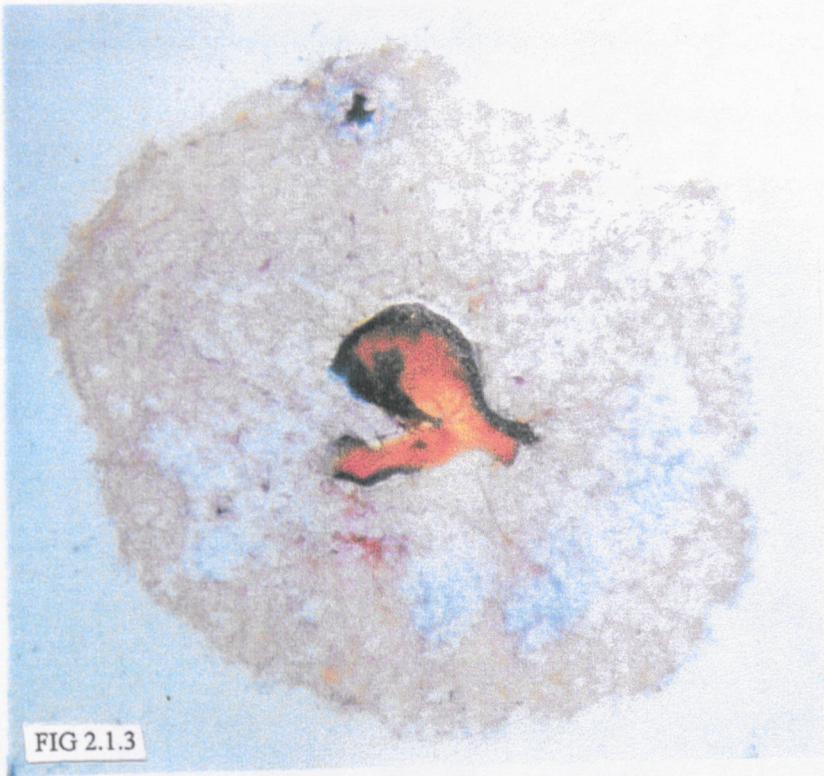


FIG 2.1.3

By comparison, non-migratory, squamous, stratified keratinising epithelium shows an even distribution of keratin.

**In spite of meticulous hygiene, during the collection of donor blood for the purposes of transfusion, an average of 20 commensal skin bacteria are released during a needle puncture (Myhre and Goldstein 1974). Fortunately, however, by using an unhurried as well as thorough approach and cleansing with several iso-propyl swabs in a brisk manner (Boxall 1976), there is a good neutrophil response (Boxall et al., 1980). The presence of neutrophils in the explants and addition of antibiotics at low concentrations ensured that every effort was made to obtain viable, bacteria-free cultures.**

### **2.2.1 LEISHMAN STAINING METHOD (Used in Chapter 3)**

The membranes were rinsed in several changes, depending upon the presence of red cells, of Dulbecco's modification of Eagle's Medium (DMEM) and fixed overnight in acetone-free methanol. More prolonged fixation rendered them too brittle. They were then immersed in Leishman's stain (0.15% in methanol) diluted 1:5 with phosphate buffer pH 6.8 and mixed, prior to use, by inversion or pipetting. The staining process was evaluated at hourly intervals using microscopy and, when the stain had penetrated the membrane, the cells were differentiated by rinsing in pH 6.8 buffer. It was found important to immerse the membranes properly and to avoid contact with the surface sheen of the staining mixture in order to avoid the presence of staining precipitate in the final preparation. Following differentiation, the membranes were briefly dehydrated in ethyl alcohol, cleared in cedar wood oil and mounted in neutral balsam.

### **2.2.2 HEIDENHAIN'S METHOD**

**Foetal tympanic membranes and segments of adult membranes were washed in DMEM and fixed, overnight, in Zenker's fluid:**

**Potassium dichromate 2.5g**

**Mercuric chloride      5g**

**Distilled water      100ml**

**Glacial acid      5ml added just before use**

**On the following day, the membranes were washed in running tap water and immersed in 5% ammonium ferric sulphate in distilled water ( i.e., mordant), for four hours and stained with Heidenhain's haematoxylin:**

**Haematoxylin 1g dissolved in 19ml ethanol + 90 ml**

**distilled water, stoppered and ripened for a few days.**

**The membranes were differentiated in this mordant, dehydrated in graded alcohols (70-100%), cleared in cedar wood oil and mounted in balsam.**

### **2.3.1 COVERSIP CULTURES EMBEDDED IN EPOXY-RESIN FOR EXAMINATION BY LIGHT AND TRANSMISSION ELECTRON MICROSCOPY (CHAPTER 4)**

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The coverslip cultures were fixed in 2.5% glutaraldehyde in sucrose-cacodylate buffer overnight. This fixative was prepared on a monthly basis in a fume cupboard, as follows:-

0.2 M aqueous sodium cacodylate      33 ml

25% glutaraldehyde (stock solution)   20 ml

Distilled water                              47 ml

pH adjusted to 7.2 - 7.4 using 0.1 M HCl and the solution converted  
to sucrose cacodylate buffer:

0.2 M aqueous sodium cacodylate solution   100 ml

Sucrose 7 g

3) Immersed in a mixture of 2% Osmic acid + 2% buffer 50:50 (=1%), for 1 hour (the stock solutions of osmic acid and buffer were stored at 5°C).

2% buffered osmium tetroxide (osmic acid) for post fixation:

Veronal acetate buffer, stock solution:

Sodium barbitone                      2.94 g

Sodium acetate (anhyd)              1.15 g

Distilled water                          100 ml

Working solution:

Veronal acetate buffer stock solution   5 ml

0.1 M HCl                                      5 ml

Distilled water                              20 ml



- 4) Immersed for 10 minutes in three changes of distilled water.
- 5) Immersed for 10 minutes in ethyl alcohol diluted with sterile distilled water: 30%, 50%, 70%\*, 85%, 95% and three changes in 100% \*(the process could be safely delayed for several days in 70%).
- 6) Immersed in two changes of propylene oxide, using a glass container, each for 15 minutes.
- 7) Immersed in a mixture of propylene oxide and epoxy resin 3:1 for 2 h.
- 8) Embedded in epoxy resin, kept at 60°C for 2-3 days, until hard enough for microtome cutting.

I cut serial sections, with an interference colour of light to medium yellow to give good contrast at low magnification, on an ultramicrotome (Reichert Jung Ultracut E). The embedded cholesteatoma culture of *en masse* migrating epithelium was cut starting at its leading edge.

Prior to examination using light microscopy, the sections were placed on separate drops of distilled water on a microscope slide, dried and stained on a hot plate at 60°C using filtered (Whatman No 1) toluidine blue:

Borax 1 g dissolved in 100 ml distilled water

+ Toluidine blue 1 g

Sections used for examination by transmission electron microscopy were stained using uranyl acetate:

Discrete drops of uranyl acetate were placed on a small sheet of dental wax in a water saturated atmosphere. This was accomplished by simply bending down the four corners of the wax sheet and placing it on a filter

paper moistened with distilled water, in a covered petri dish. Sections mounted on grids were placed, face down, on the surface of the droplets and stained for thirty minutes, rinsed in distilled water, placed face down on a drop of saturated lead citrate for 10 minutes and finally rinsed again in distilled water and allowed to dry.

## CHAPTER 3 THE MORPHOLOGY AND STRUCTURE OF THE TYMPANIC MEMBRANE *IN VITRO*

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### 3.1.1 INTRODUCTION

At the Mastoid and Cholesteatoma Conference in 1988 (Proceedings published 1989), Abramson described his use of cartilage blocks as a substitute for the loss of elastic fibres in the pars flaccida, in order to prevent in-growth of skin and to act as a barrier between epidermis and connective tissue or granulation tissue; after atticotomy or canal up mastoidectomy and to eliminate post operative retraction pockets.

He made the bold statement *'contraction of myofibroblasts within granulation tissue induces cholesteatoma through epithelial migration by reorientating connective tissue fibres in a medial direction providing contact guidance to medial direction of keratinocytes'*.

If this is so, then disruption of the radial connective tissue fibres of the TM can be expected to affect the migration of the adjacent, epithelial strata on the surface of the tympanic membrane.

In vitro, the established migration of colonies of epithelium, prepared by trypsinisation and cultured on irradiated 3T3 Swiss mouse fibroblasts, is linear (Boxall 1993). The en masse migration of epithelium from explants is similarly linear.

There are occasions when migration is not linear. One of these occasions is following death of some of the cells in a sheet of epithelium. The spaces created by the loss of cells encourages mitoses in surrounding cells, by

loss of contact inhibition. As dead epithelium is shed, the dividing cells expand into that space so that strict linearity of movement is lost. I have observed, in time-lapse recordings, that this sequence of events is frequently repeated at the same foci (unpublished).

However, it has been established that, in vivo, the normal en masse migration of epithelium from the umbo to the periphery of the tympanic membrane is a phenomenon most probably regulated by mitoses (Smelt *et al.*, 1986). In vitro, the direction of growth and the polarity\* of individual epithelial cells of the tympanic membrane and most cholesteatoma epithelium is the same as the direction of en masse migration of the surface of the TM .

\*(Cultured epithelial cells seen in the matrix during en masse migration which are oval in shape can be described as having an axis and therefore polarity.)

If migration is interrupted by an obstacle e.g., a tube or grommet inserted for the purposes of ventilating or draining the middle ear, then the flow of epithelium is temporarily interrupted and epithelial cells may pile-up and thicken at the point of contact. Following perforations from injury or infection, which cause deep penetration and damage to the connective tissue layers of the tympanic membrane, more prolonged and even permanent changes in the direction of migration may result.

It was suggested by Abramson that changes in the layers deep to surface epithelium could influence the direction of growth of the surface squamous epithelium. This has major implications for the management of

cholesteatoma and suggests that once damage occurs to deeper layers of the TM then the growth of cholesteatoma may be almost inevitable.

The object of this part of the thesis was to see whether disruption of the radial fibroblast layer of the TM was related to changes with the patterns of polarity of the surface epithelial cells. Any related changes would support Abramson's hypothesis.

### 3.1.2 METHOD

During my search for an interrelation between the polarity of the radial fibroblast layer and the direction of migration of the adjacent epithelial layer of the tympanic membrane, I found that it was impossible to identify and to follow the basement membrane which is the dividing line separating these layers because it is tortuous and frequently strays outside the confines of serial microtome sections. Close attention to gross dissection, fixation, dehydration and orientation during embedding and section cutting does nothing to solve this problem.

I therefore stained whole human foetal tympanic membranes and, using light microscopy, focussed and examined the structure of each strata in the membrane. The human foetal tympanic membranes used in this experiment grow very early in foetal life and by the end of the second trimester, which I determined by the length of the whole foetus (see table in the Appendix), they are almost adult size.

Pieces of adult post-mortem tympanic membrane were stained, in addition to the whole, fresh, foetal tympanic membranes. Following harvesting the membranes were stored in Dulbecco's modification of Eagle's

medium (DMEM) at 4 to 6° C. Other studies showed that pieces of post-mortem membrane proved viable up to six days following death, providing that the tissue was not contaminated and was free from infection. The shapes of segments or pieces of membrane were sketched and their approximate location on the ear drum recorded.

An optimum stain for this purpose needed to impregnate each layer of the tissue, differentiate the cellular content for identification and assessment of their overall polarity, without impeding the source of illumination necessary for photomicrography and for focussing at different levels within a thick specimen.

Several fixatives and stains were tested and two were found suitable to meet these requirements.

The first, a Romanowsky stain using Leishman's method (see 2.2.1.) is primarily used to stain and differentiate blood films. This penetrated tympanic membranes, stained the cell components throughout the matrix and could be differentiated to allow the passage of sufficient intense light for microscopy and photography.

Both staining methods gave equally good staining. Using intense light microscopy, the first layer of cells in the membrane were identified and recorded by photography. Then, retaining the same field of vision, the focus was readjusted until cells in the next stratum were identified (Fig 3.1.2) and photographed. This process continued until each layer of the membrane was recorded (Figs 3.1.2 - 3.1.8). The number of cells in each layer was quantified by counting those in focus where the stratum was most dense. A simple

comparison, in vitro, between the difference in the angle of the cells was recorded in degrees, using the vertical axis of the field of view as a base line (i.e., zero<sup>0</sup>). The depth of each field was measured using the calibrated fine adjustment of the Nikon Optiphot microscope (X 100 objective: 1 division = 1  $\mu$ m). The overall thickness of treated membranes was different to fresh membranes, therefore these measurements could only be used for the purposes of simple comparison between the different layers.

### 3.1.3 RESULTS

The surface keratin had been washed away during the staining process in all specimens. Eight distinct layers could be detected in every specimen (Figs 3.1.1 - 3.1.8 and Table 2). There were no differences between mature foetal and adult membranes.

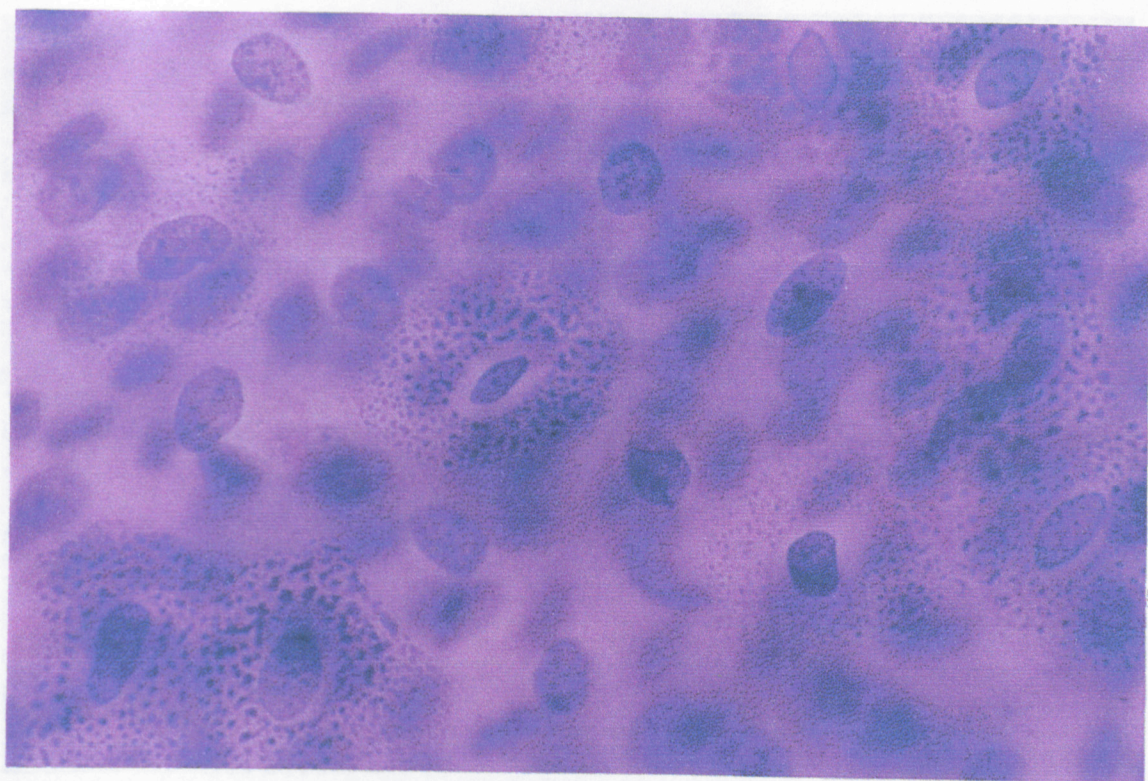
#### FIRST LAYER (Fig 3.1.1):

This layer corresponded to the *granular layer* with a few keratinised epithelial cells from the superficial *stratum corneum*. Cytoplasm in a few of the granular cells contained keratin granules, precipitated during fixation, and their oval nuclei were condensed. Overall, most of the cells showed no polarity and where polarity was present they shared no angle common to one another.

I measured the thickness of each layer by using the graduated fine focus knob, calibrated in 1 $\mu$ m divisions, of a Nikon microscope. The thickness of the first layer measured 75 $\mu$ m. I then counted the number of epithelial cells in sharp focus at the level in this layer where their concentration was most dense; this amounted to 20 per field using an oil immersion lense.

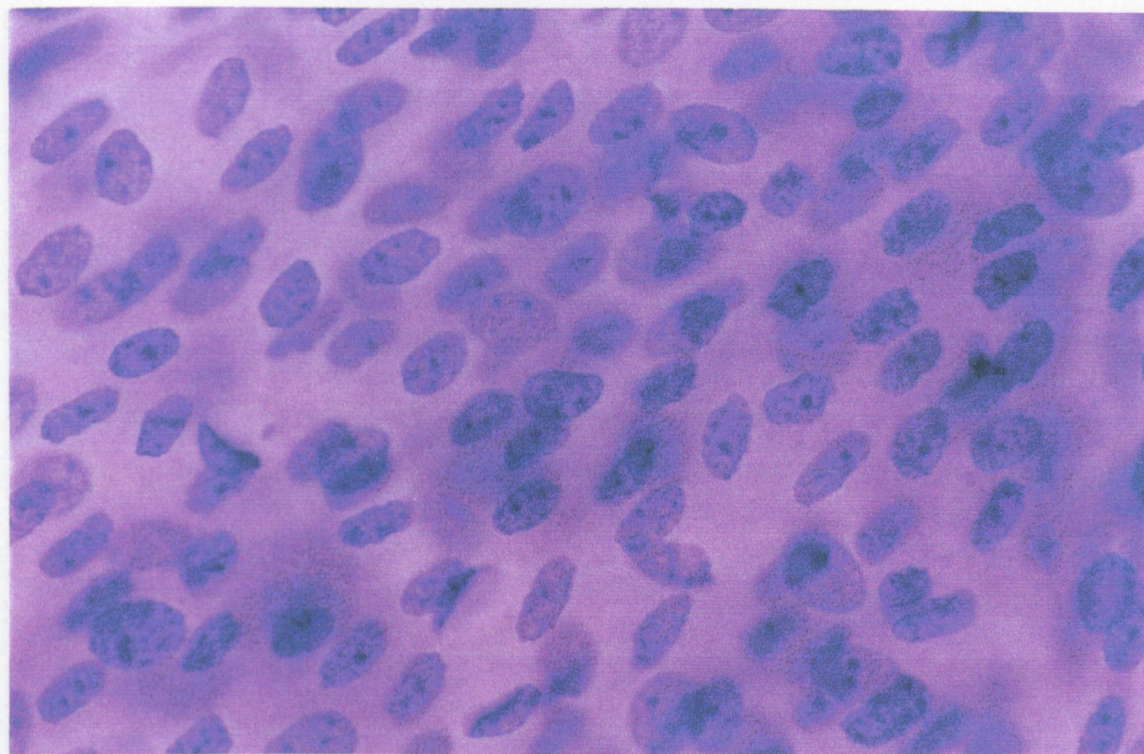
#### SECOND LAYER (Fig 3.1.2):





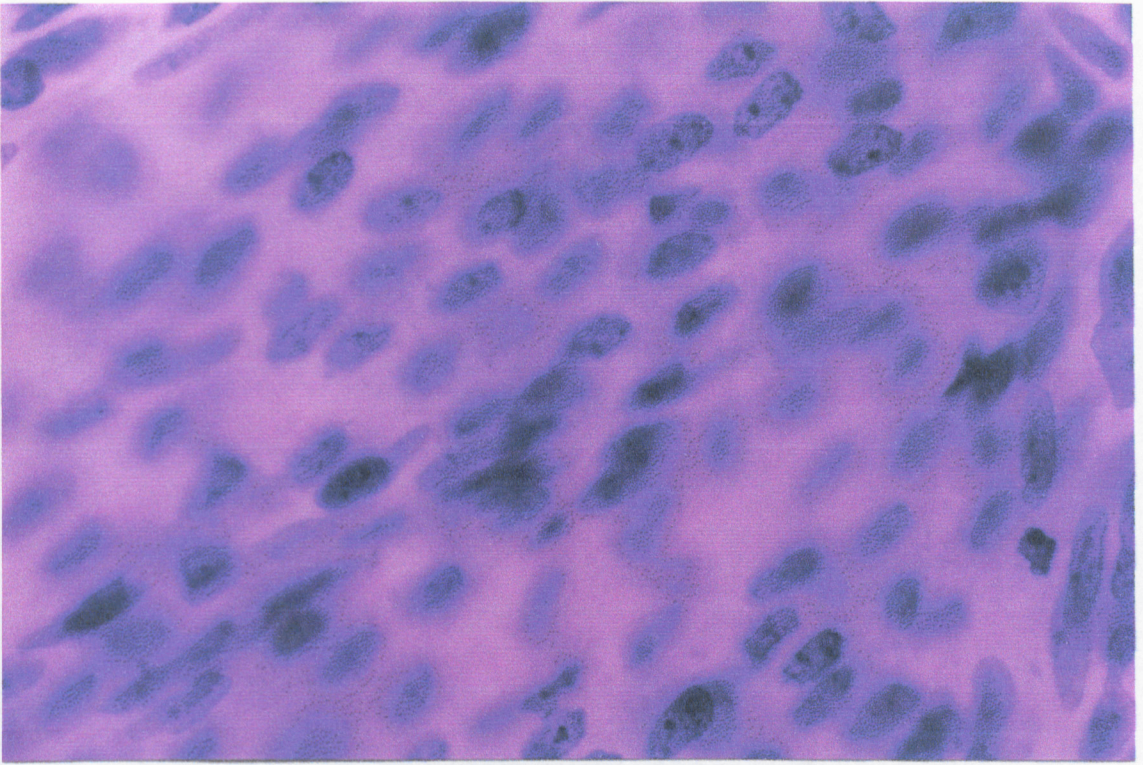
**Fig 3.1.1: Granular layer with remnants of the Stratum corneum**

100μm

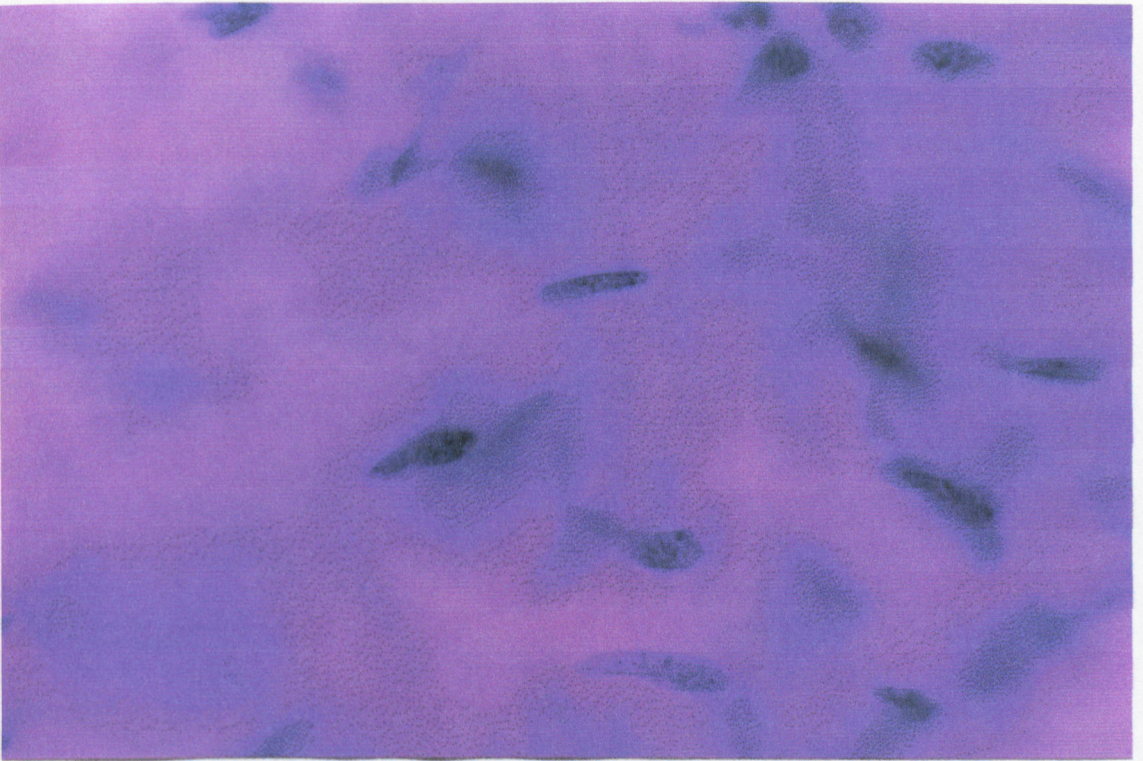


**Fig 3.1.2: Prickle layer, axes of the oval cells + 45° from the vertical**



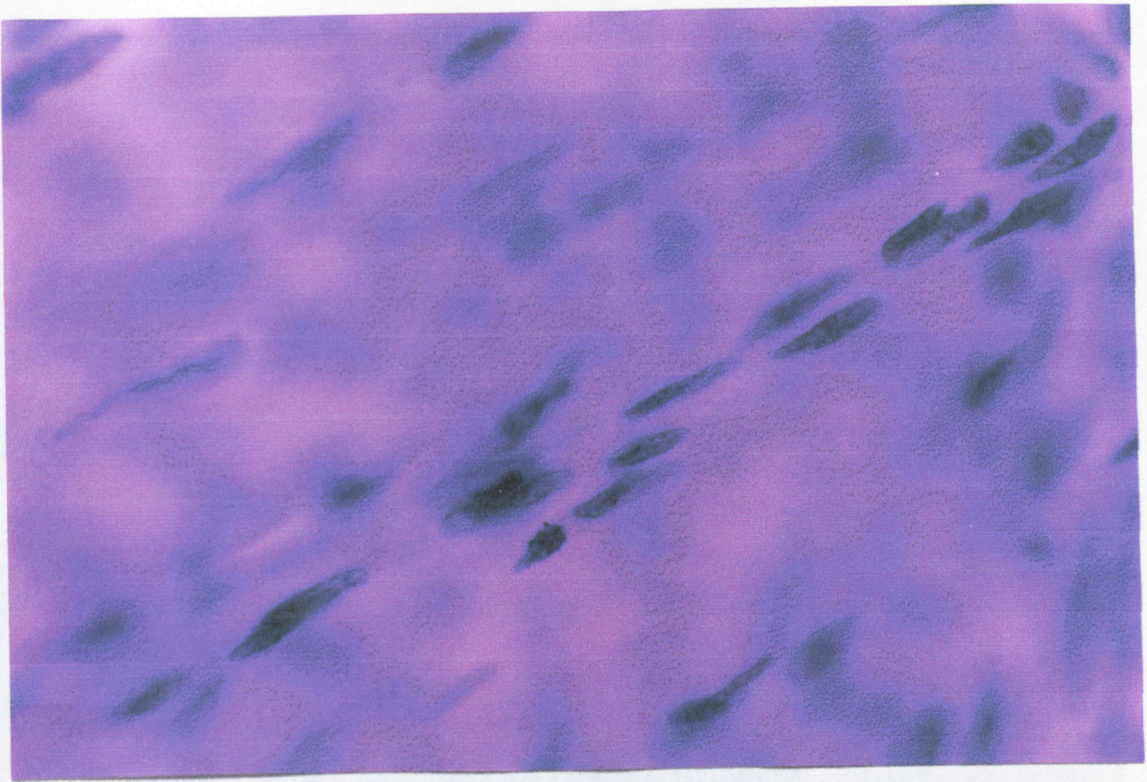


**Fig 3.1.3: Prickle/Basal layer. Polarity: no change from the previous layer**

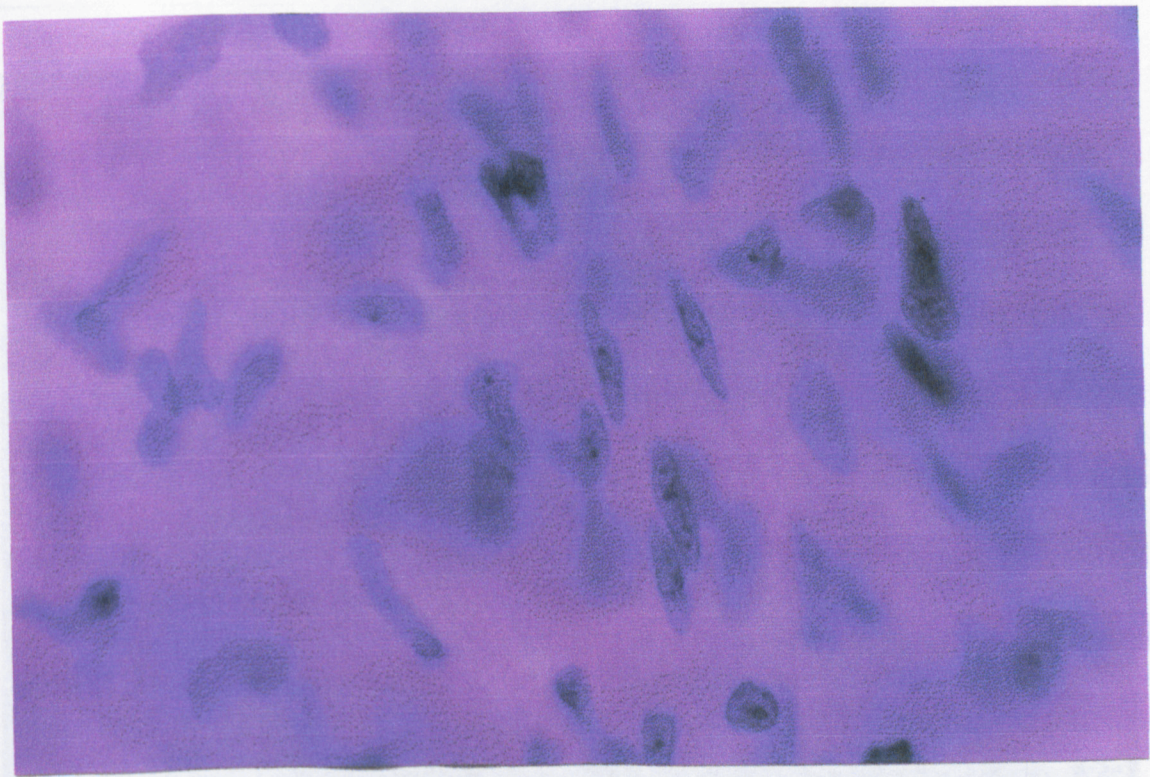


**Fig 3.1.4: Fibroblasts. Polarity +45° (90° from Fig 3.1.1). Individual cells spaced well apart**



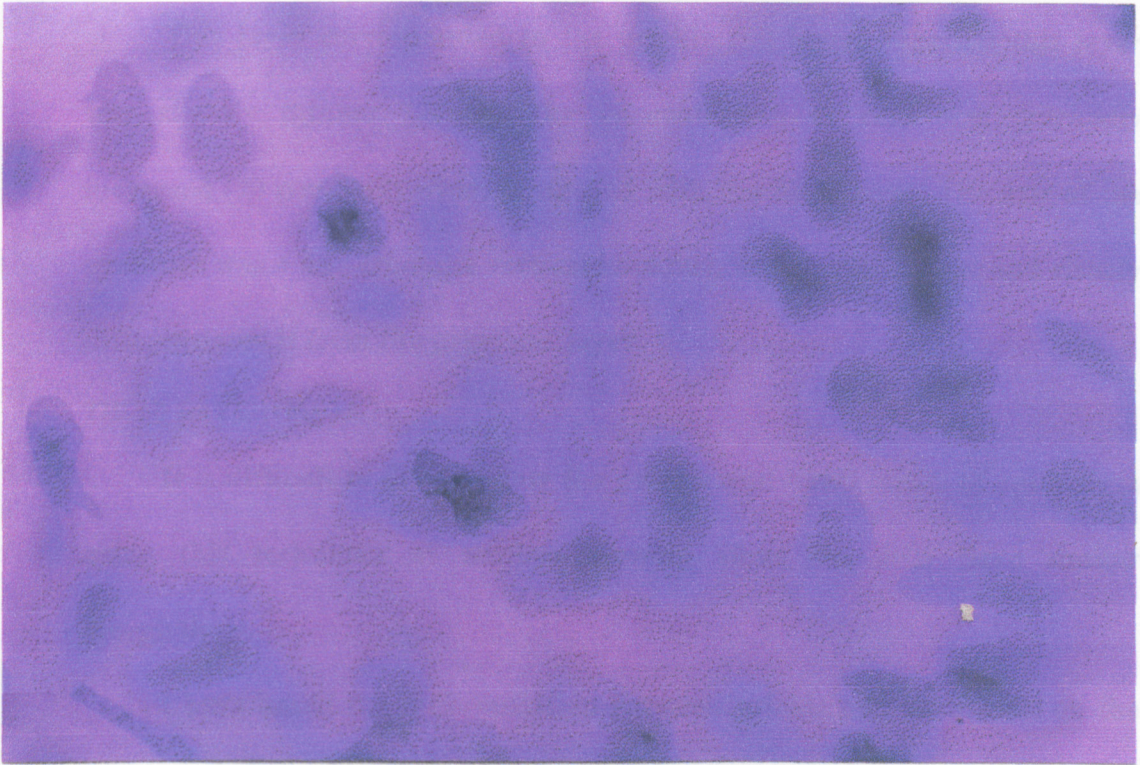


**Fig 3.1.5: Layer of radial fibroblasts +45° from the vertical, in twin column arrangement**

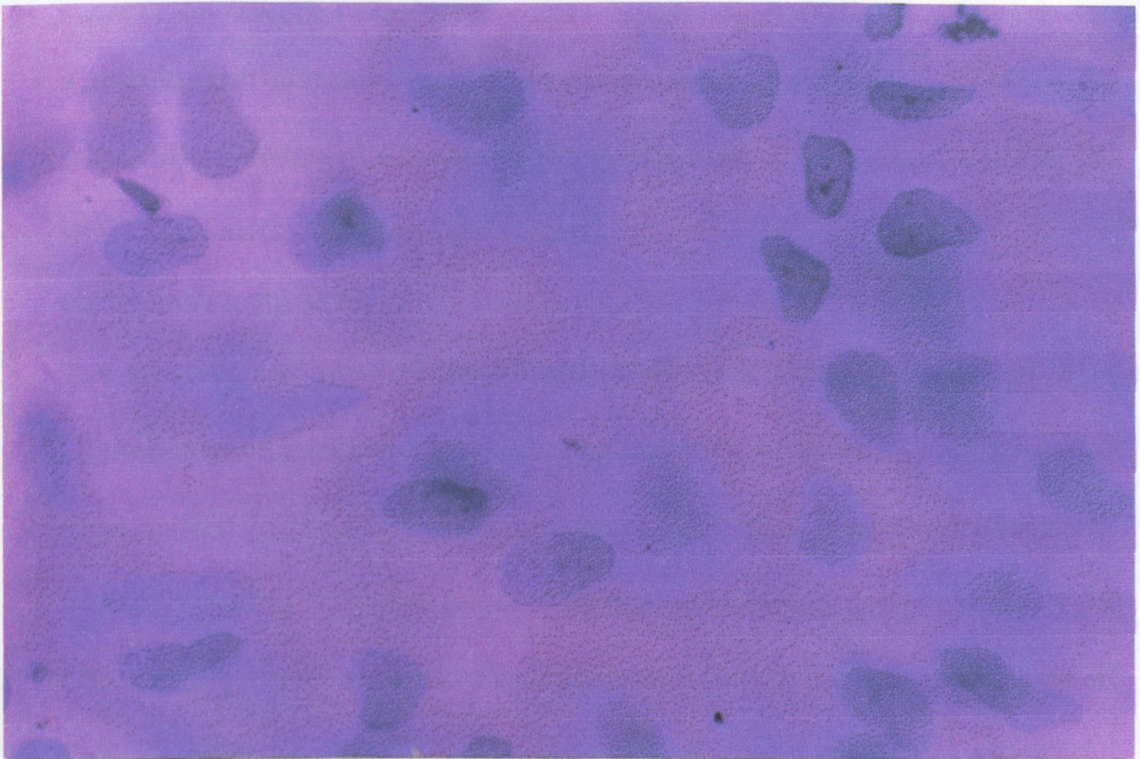


**Fig 3.1.6: Circular layer of fibroblasts, axes a few degrees from the vertical**





**Fig 3.1.7: Connective tissue containing a few fibroblasts, no common axes**



**Fig 3.1.8: Middle ear epithelium, no common axes**

This layer corresponded to the prickle cell layer. Whereas this layer was slightly thinner ( $70\mu\text{m}$ ), the concentration of cells was markedly increased (70 per field) when compared with the first layer. The axis of the oval cells was  $+45^{\circ}$  from the vertical base line, in the *prickle layer* and clearly differentiated it from the previous layer.

#### THIRD LAYER (Fig 3.1.3):

It is difficult to differentiate the prickle and basal layers of epithelium using this technique of surface viewing since the cells have similar morphology.

I have based the differentiation between prickle and basal cells on the proximity of the basal layer to the underlying fibroblasts which are clearly discernible. The basal layer is  $55\mu\text{m}$  in depth with 50 cells per field, the axis of the oval cells was  $+45^{\circ}$ .

#### FOURTH LAYER (Fig 3.1.4):

This thin layer ( $45\mu\text{m}$ ) of connective tissue contained a sparse population (10 per field) of elongated fibroblasts showing a further increase of  $+45^{\circ}$  in the angle of polarity away from the vertical.

#### FIFTH LAYER (Fig 3.1.5):

The formation of some of the fibroblasts (13 per field), in the radial layer ( $30\mu\text{m}$ ), was of particular interest. The direction of polarity ( $+45^{\circ}$ ) was the same as that in the third layer, i.e., basal epithelium, and these fibroblasts were arranged in "twinned column" formations whereby two parallel rows of fibroblasts formed a strand-like appearance running across the plane of focus.

#### SIXTH LAYER (Fig 3.1.6):

Wide bands of fibroblasts in the sixth layer showed a difference in the angle of the axis of the elongated cell compared to the fifth layer. The angle was a few degrees negative. The thickness of this layer was 30 $\mu$ m and the concentration of fibroblasts 17 per field. This layer corresponded to the circumferential layer of fibroblasts.

**SEVENTH LAYER (Fig 3.1.7):**

There was no common direction among the scanty population of fibroblasts (7 per field) in this layer of connective tissue (30 $\mu$ m thick).

**EIGHTH LAYER (Fig 3.1.8):**

This consisted of a thin (20 $\mu$ m), scanty (15 per field) layer of middle ear epithelium, only a few showing polarity and sharing no common direction.

**3.1.4 DISCUSSION**

It has been proposed by Abramson that besides sharing the same angle, the regular arrangement of fibroblasts, in the layer adjacent to migrating epithelium on the membrane, could influence the overall direction of epithelial migration. It could be imagined that the twinned columns of fibroblasts form "rails" between which are small depressions that act as guides to the migration of the more superficial epithelial cells.

A simple analogy would be faint undulations in the flooring beneath a carpet affecting the path of a ball rolled across its surface.

However, this proposal, would lead to an extra depth of epithelial cells in the grooves which was not observed in any of the specimens. It is therefore difficult to sustain this "mechanical" model of epithelial migration.

Furthermore, after finding areas where the polarity of the fibroblasts layer appeared irregular, disrupted or misaligned, I failed to demonstrate

successfully any corresponding changes in the direction of migration of the adjacent epithelium. This strongly suggests the direction of migration of the epithelium is not related to the arrangement of underlying fibroblasts at least in the pars tensa.

It is therefore difficult for Abramson's suggestion of a direct mechanical influence on epithelial migration to be sustained.



TABLE 3: STRATIFIED LAYERS OF THE TYMPANIC MEMBRANE

LEVEL, DEPTH, N°CELLS, DESCRIPTION, ANGLE, CORRESPONDING STRATUM  
(in focus) (from vertical)  
↓

(External surface of the tympanic membrane)

EPITHELIUM

1	75um	20	(oval nuclei remnants, and condensation of the cytoplasm)	↓ none: ↓ none:	Stratum corneum remnants and Granular layer
---	------	----	--	--------------------------	--

2	70um	70	(oval cells)	+45°: ↓	Prickle layer
3	55um	50	(oval cells)	+45°:	Basal layer

FIBROBLASTS

4	45um	10	Sparse, elongated.	↓ + 90°: ↓	Thin layer connective tissue
5	30um	13	Twinned column arrangement.	↓ + 45°: ↓	Radial layer of fibres
6	30um	17	In wide - few degrees: bands.	↓ ↓	Circular layer of fibres
7	30um	5		none:	Connective tissue

EPITHELIUM

8	20um	15		none:	Middle ear epithelium
---	------	----	--	-------	-----------------------

NB: Layers 3 and 5 share the same angle

## CHAPTER 4

### THE MORPHOLOGY OF TYMPANIC MEMBRANE AND CHOLESTEATOMA EPITHELIUM PRIOR TO *EN MASSE* MIGRATION *IN VITRO*

---

#### 4.1.1 INTRODUCTION

I have previously reported the difference between abundant outgrowths of epithelium obtained in cultures of explants dissected from the neck of a cholesteatoma sac (Boxall 1993).

This chapter of my work now examines the structure of cholesteatoma epithelium before *en masse* migration takes place.

#### 4.1.2 MATERIALS AND METHOD

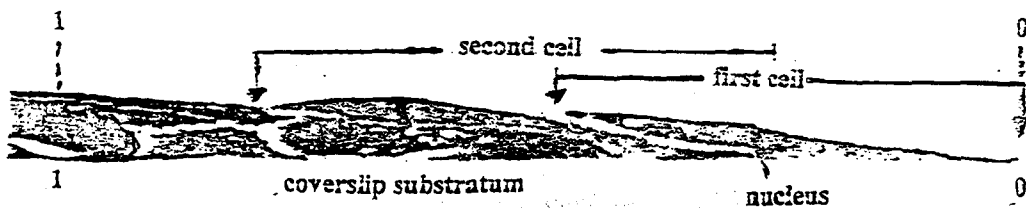
Fragments of cholesteatoma tissue were obtained from adults with acquired cholesteatomas, at surgery. The epithelium was harvested, prior to *en masse* migration, using fine pointed forceps to remove and gently separate the sandwich coverslips. These were briefly rinsed them in three changes of sterile DMEM to remove any serum which might precipitate during fixation. The coverslip cultures were fixed in glutaraldehyde and stained for examination by light and electron microscopy (see chapter 2: 2.3.1).

#### 4.1.3 RESULTS

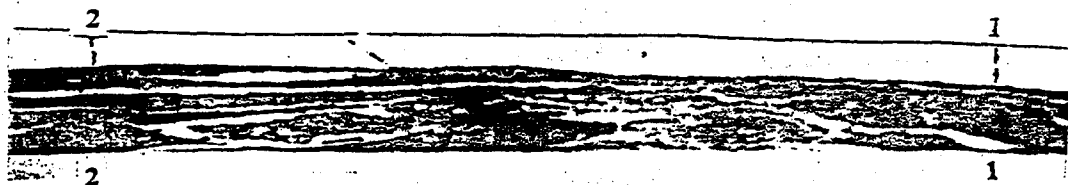
I found no differences in the morphology of tympanic membrane and cholesteatoma epithelium examined by light microscopy ( Fig 4.1.1 ).

Throughout each stratum, there is a considerable overlap of the epithelial cells. Half of the ventral surface of the first cell is in contact with the coverslip substratum and the other half overlaps the second cell, of which

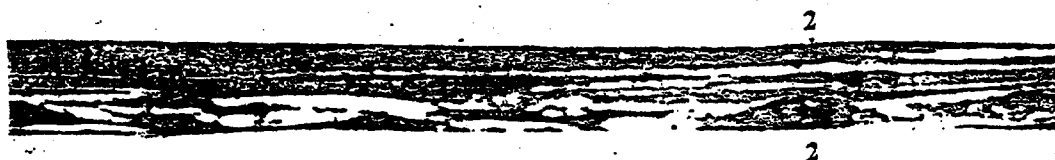
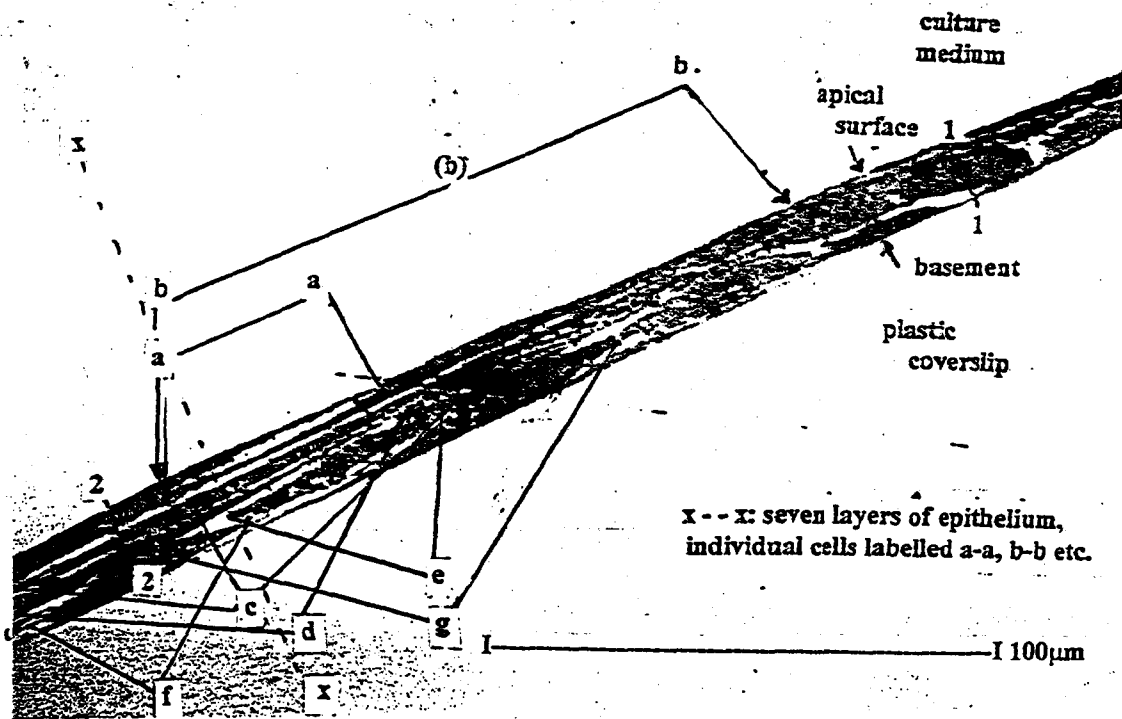




Section of an outgrowth of epithelium from a cholesteatoma explant prior to migration. This shows the overlap of first two cells and upturned lamellae at the leading edge. There is close contact between the basal layer of the cells and the coverslip.



In this adjacent section there are up to seven layers of cells, some partly exposed on the surface 'b - b' have a diameter in excess of 100 $\mu$  (detailed below).



In the third section, closer to the cholesteatoma explant, there are less contact points with the substratum and between the layers of epithelium.

Fig 4.1.1 MORPHOLOGY OF TYMPANIC MEMBRANE AND CHOLESTEATOMA EPITHELIUM PRIOR TO EN MASSE MIGRATION IN VITRO

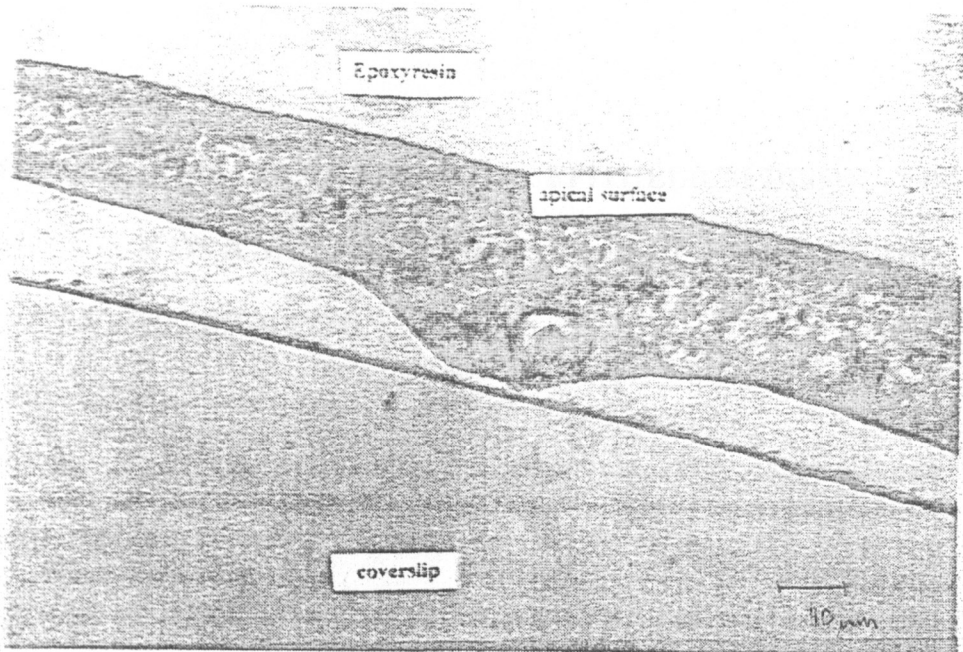
about one third is in contact with the substratum and two thirds is in contact with the apical surface of the third cell.

The extent of this overlap obviously depends upon the location of the cut in relation to a surface area in each cell, some close to their perimeters, others showing cut nuclei. The cholesteatoma matrix, in the section illustrated, reached a maximum thickness of seven cells (a - g), some of which, close to the surface, covered a large area (diameter b-b:  $>100\mu\text{m}$ ).

Whereas, prior to migration, there is close contact between the basal surface of migrating epithelium and the coverslip substratum (Fig 4.1.1), there is sparse contact during active migration (Fig 4.1.2).

A close examination of the tip (0-0) of the leading epithelium lamellae, shows this to be upturned. Additional lamellae (Fig 4.1.3) are located on the apical surface of the leading edge (Boxall et al., 1990).

Fig 4.1.2



This section of cholesteatoma epithelium shows that there is often only minimal contact between the basal layer and coverslip substratum during periods of en masse migration

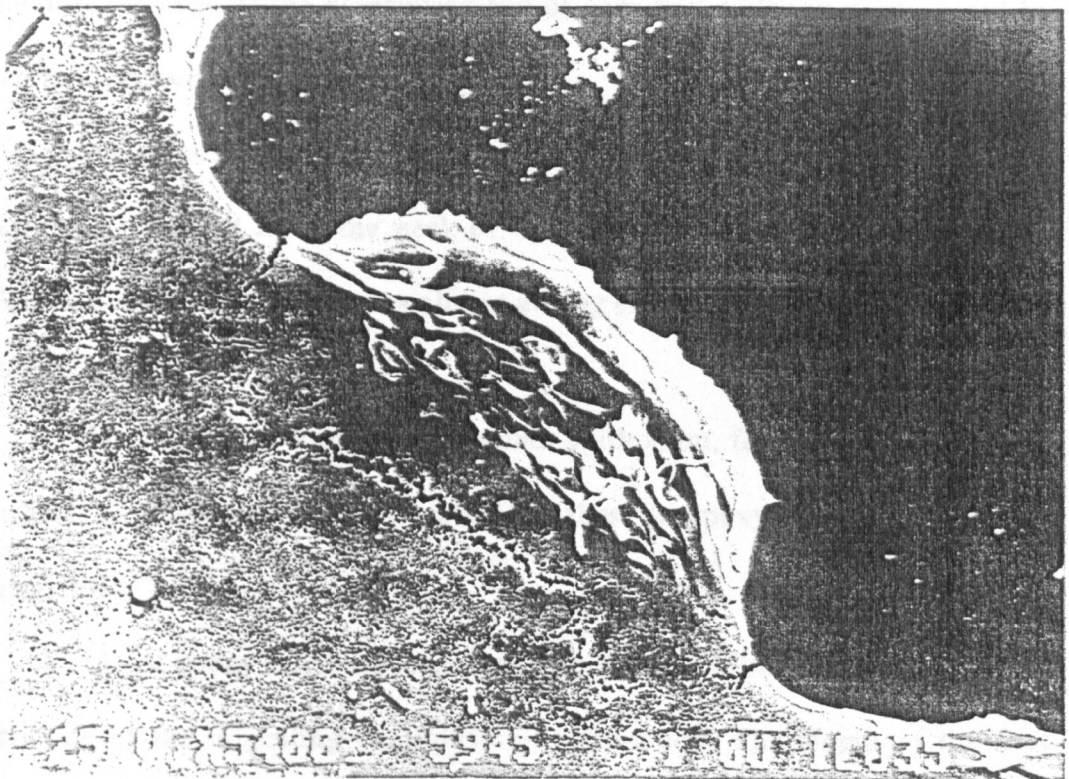


FIG 4.1.3

Multiple lamellipodia present on the surface of the leading epithelium during en masse migration  
(Boxall et al., 1990)

## CHAPTER 5

### THE STRUCTURE OF CHOLESTEATOMA EPITHELIUM DISSECTED FROM THE NECK OF A SAC, CULTURED AND SECTIONED *IN VITRO* DURING *EN MASSE* MIGRATION.

#### 5.1.1 INTRODUCTION

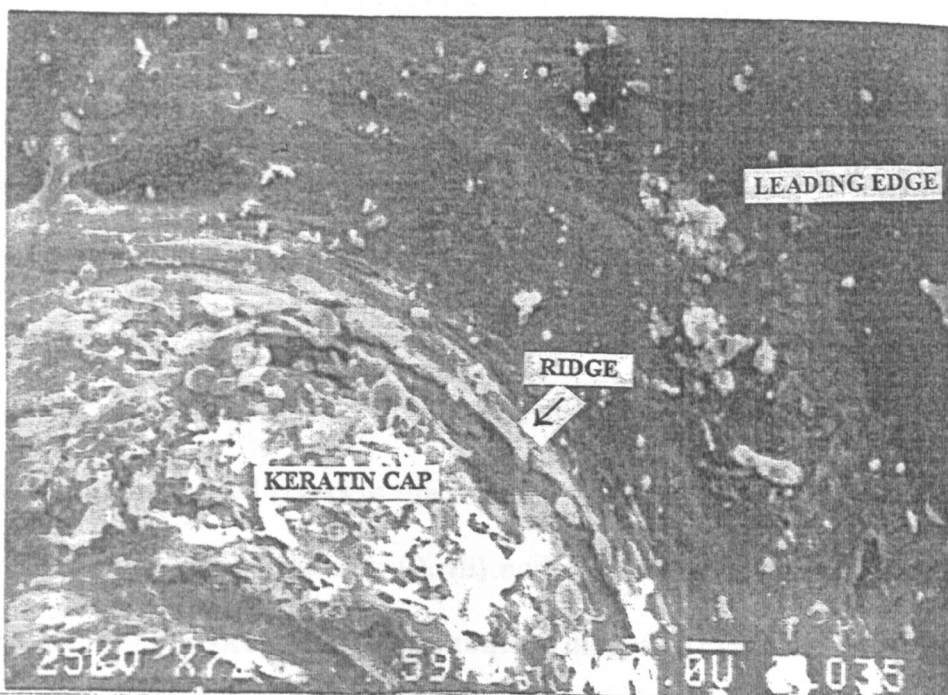
Cultures of tympanic membrane epithelium and cholesteatoma epithelium, with the exception of those derived from the neck of a sac, produce a *ridge* of spent keratinised epithelium during *en masse* migration (Figs 1.1.5 & 5.1.1). This ridge is located close to the leading edge and consists of keratinised epithelium which becomes detached from the leading edge and rolls back over the *en masse*, migrating matrix to form a ridge against the edge of the keratinised cap of cornified epithelium, produced by vertical maturation of the basal layers.

My object, here, was to examine the leading edge of cholesteatoma cultures dissected from the neck of cholesteatoma sacs, where the *ridge* of keratinised epithelial cells was absent during *en masse, in vitro* migration.

#### 5.1.2 METHOD

*En masse* migrating cholesteatoma epithelium, from the neck of a sac, where no *ridge* existed, were embedded these in epoxy resin, as previously described (2.4.1).

I cut epoxy resin sections until I could determine the presence of the leading of the migrating cholesteatoma epithelium. The length of the first section, which measured 243 $\mu$ m (Table 3) and subsequent sections cut at



**FIG 5.1.1** Scanning electron microscopy: tympanic membrane and cholesteatoma epithelium (except for epithelium dissected from the neck of a cholesteatoma sac) produce a 'ridge' of keratin. This ridge consists of dead epithelial cells, from the leading edge during *en masse* migration, which collect together in front of the keratin cap and lie parallel with the leading edge (see also fig 1.1.5).

50  $\mu\text{m}$  intervals, were stained with toluidine blue. The dimension of each section was determined by using a microscope slide graduated in microns and a graduated eye piece. These dimensions were determined:

- (i) total length of the cut section in  $\mu\text{m}$ .
- (ii) length of lamellae in  $\mu\text{m}$ .
- (iii) the presence of the following structures: vacuoles (v), nucleus (n), in the basal layer (n/b), middle layers (n/m) and surface layer (n/s), and mitosis (m).
- (iv) the thickness of each section at zero and 100 $\mu\text{m}$  intervals.
- (v) as the number of cell layers in the stratum

I correlated measurements (i), (ii) and (iv) (Table 3) and produced a three dimensional representation and contour diagram (Figs 5.1.2 & 5.1.3).

#### 5.1.3 RESULTS

In the three dimension reconstruction and contour map, the ridge is replaced by mounds and peaks of epithelium.

#### 5.1.4 DISCUSSION

The orderly formation of a ridge, between the leading edge and cap, during *en masse* migration, has been replaced by undulating mounds and peaks of epithelium. This suggests that the migratory pattern of epithelium from the neck of a sac is disorderly. At the same time, the overall velocity and direction of *en masse* migration is not affected, therefore these changes must occur in the suprabasal layers. Fragmentation of the keratin cap in some cholesteatoma cultures has been reported by Proops (1983).

The presence of epithelial peaks in the contour representation suggest that epithelium in the suprabasal layers actively migrate or are carried along,

albeit whirling around an axis. This phenomenon is further investigated, using video time-lapse recordings, in Chapter 6.

table 4

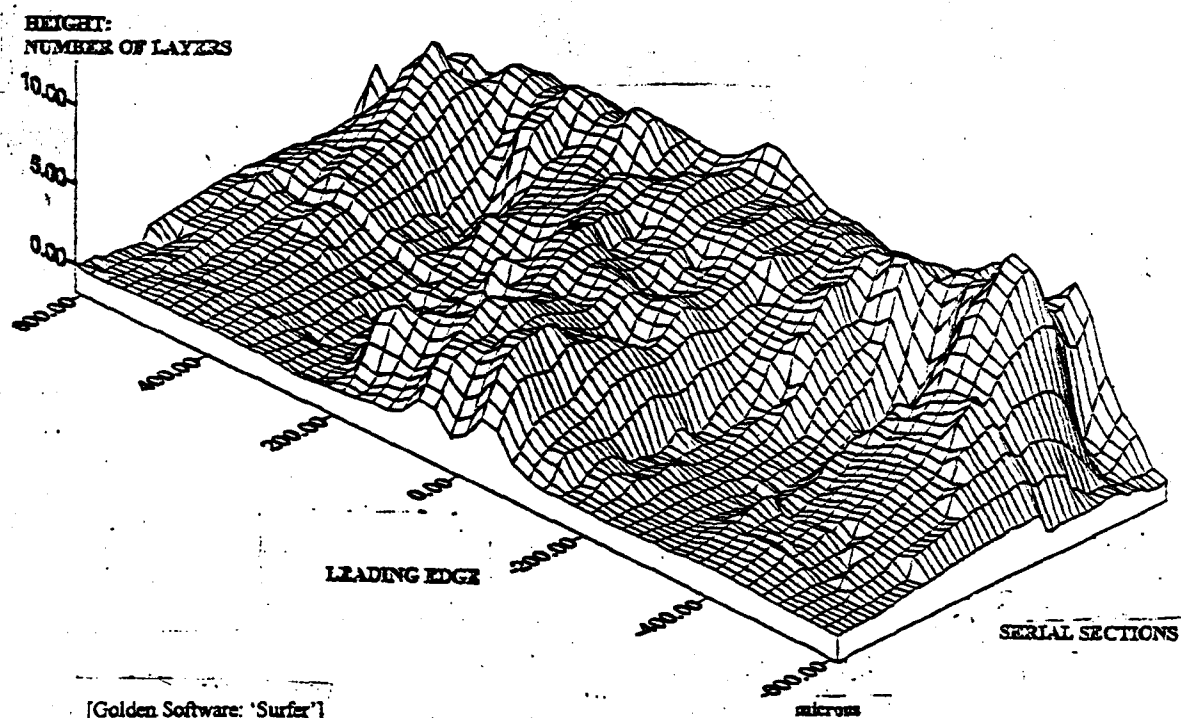
CHOLESTEATOMA CULTURE SECTIONS cut at intervals of 50  $\mu\text{m}$

Section (i) (ii) (iii) (iv):(v)  
N°  $\mu$   $\mu$   $\mu$

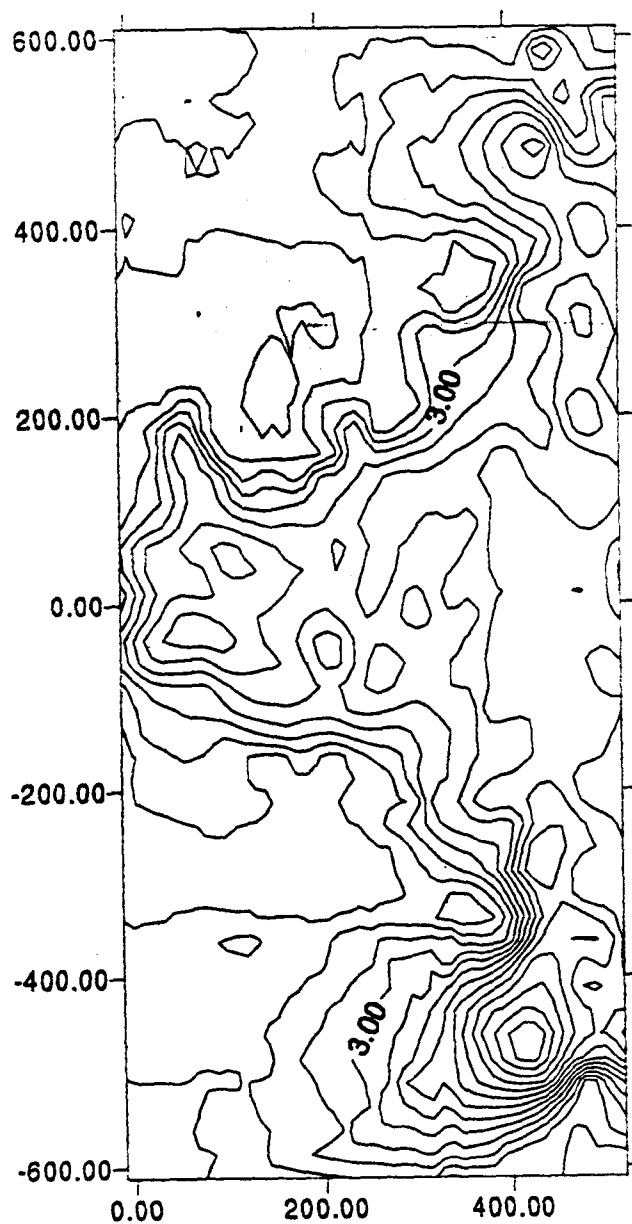
1	243*	20	n	<1:1 3:1 2:1
2	300			2:1 6:2 5:3 1:1
3	400	100	v	<1:1 2:1 5:2 2:1
4	450	100	v	<1:1 2:1 7:3 5:3 6:2
5	350			<1:1 3:2 4:3 5:2 2:1
6	300		n(2)	7:2 7:3 3:2
7	450			<1:1 3:1 5:3 5:3 <1:1
8	300			1:1 5:2 6:3 2:2
9	300			2:1 5:2 5:3 1:1
10	350			1:1 5:3 5:3 2:2
11	300			1:1 2:1 5:3 4:2
12	300			1:1 2:1 3:2 2:1
13	450			1:1 4:2 5:2 5:3 6:3 1:2
14	350		n	1:1 6:3 6:3 5:3 2:1
15	500			2:1 2:1 5:3 5:2 5:3 1:1
16	400			3:2 4:3 3:2 6:3 1:1
17	630		nml	<1:1 5:3 4:3 4:3 4:3 3:2 3:2 <1:1
18	600		nmit	<1:1 4:2 5:3 6:3 6:3 4:3 8:1 <1:1
19	620			<1:1 6:3 4:3 4:3 4:3 4:2 1:1
20	630		vnbms	<1:1 7:2 7:4 6:4 6:3 5:2 <1:1
21	700			<1:1 3:2 5:3 7:3 7:3 7:3 4:2
22	1150			<1:1 14:3 9:5 10:3 6:3 6:3 6:3 6:3 5:3 7:2 5:2 5:2
23	1220			<1:1 9:3 10:3 8:3 8:2 6:3 6:3 7:2 4:3 4:3 3:2 8:2 5:2
24	1080			<1:1 9:3 9:3 8:3 7:3 7:3 7:3 5:2 6:3 5:2 5:2
25	1000			5:2 6:2 5:3 5:3 7:3 6:2 5:2 9:2 8:3 7:3
26	1150			<1:1 14:3 9:5 10:3 6:3 6:3 6:3 6:3 5:3 7:2 5:2 5:2
27	1120			<1:1 9:3 10:3 8:3 8:2 6:3 6:3 7:2 4:3 4:3 3:2 8:2 5:2

N.B. Section 1 243  $\mu\text{m}$  was the first section, close to the leading edge which was seen to contain epithelium. (i) was the length of each section, (ii) length of the leading lamellae, (iii) structures present: v=vacuoles, n=nuclens seen in the b-basal, m-middle and s=surface layers (iv) was the section thickness and (v) the number of layers present





**Fig 5.1.2: 3D representation of cholesteatoma sac epithelium migrating *en masse*. The absence of a ridge has been replaced by mounds and pinnacles of epithelium. During section cutting it was not possible to discern the first few cells which are missing from the leading edge.**



**Fig 5.1.3: Contour map corresponding to the three dimensional representation.**

The units are  $\mu\text{m}$  and it is interesting to note the large areas covered by lamellae ( $<1 \mu\text{m}$ ) and monolayers.

## CHAPTER 6

### *IN VITRO* COMPARISONS BETWEEN THE MORPHOLOGY AND MIGRATORY CHARACTERISTICS OF TYMPANIC MEMBRANE EPITHELIUM AND EPITHELIUM CULTURED FROM THE NECK OF CHOLESTEATOMA SACS, USING TIME-LAPSE VIDEO RECORDING

#### 6.1.1 INTRODUCTION

This experiment consists of time-lapse recording differences between cultures of epithelial explants dissected from the “neck” region of cholesteatoma sacs and explants from the rest of the sac which are identical with cultures of tympanic membrane epithelium.

First, explant cultures from the neck region of cholesteatoma sacs produced very rich harvests of epithelium, while explants, selected as ‘identical and adjacent’, from other regions of the same cholesteatoma, produced ‘unpredictable’ yields of epithelium. This suggested differences in the location and distribution of mitotic ‘foci’.

Second, “solitary” epithelial cells were seen to migrate in cultures of cholesteatomatous tissue dissected from the “neck region”.

These findings were present in a cholesteatoma culture ‘C19’ from the neck of a sac and, selected as a typical representation, these cultures were recorded using time-lapse (VHS) video tape. From this recording, I prepared a 15 minute edited version, for inclusion with this thesis. The recording includes a sequence which shows the destruction of epithelial cells after coming into contact with an unidentified cell and fibroblasts.

Table 5 (C19) describes the progress of the eight explant cultures, together with their suitability and selection for video recording.

#### **6.1.2 DOCUMENTATION**

My laboratory cholesteatoma reference 'C19a/11<sup>96</sup>' denoted: 'C' = cholesteatoma, '19' = date of the month on which it was surgically removed, 'a' = the 'first' of two cholesteatomas received for culture on that date, '11' = month and '96' = year.

#### **6.1.3 CHOLESTEATOMA TISSUE: SELECTION**

Among this series of 100 cases of acquired cholesteatoma, C19 was surgically removed from a 24 year old female patient attending the Royal National Throat, Nose and Ear Hospital for mastoidectomy. It was one of a group of 5 cholesteatomas in this series producing a profuse growth of epithelium and single, migrating epithelial cells, typical of tissue explants arising from the neck of the sac. Tissue cultures were prepared within a few hours of the operation taking place.

All procedures prior to incubation of the cultures were confined to a class one safety cabinet using adequate, personal, protective clothing.

#### **CHOLESTEATOMA TISSUE: GROSS DISSECTION**

The cholesteatoma tissue, submitted for tissue culture, consisted of ragged tissue fragments collectively amounting to a few cubic millimetres.

This was transported from the operating theatre in sterile tissue culture transport medium at room temperature: Dulbecco's modification of Eagle's

Medium (DMEM) with added Penicillin 20 units/ml and Streptomycin 20 µg/ml.

The tissue fragments were briefly rinsed in three fresh changes of DMEM to remove floating dead epithelial scales and red cells. Dissection of these fragments provided sufficient epithelium for eight small explants. Each explant was sandwiched between Thermanox coverslips (5 X 22 X 0.15-0.185 mm) and these were spot welded, to hold them together, at each corner using the red hot tip of a dissecting needle. The sandwich mounted explants were then inserted into eight Nunclon plastic tissue culture flasks (each, 80 cm<sup>2</sup> area, 260 ml capacity) with vented caps and, to hold them stationary, the coverslip sandwiches were spot welded to the bottom wall of each flask.

#### GROWTH MEDIUM (Basic details contained in Chapter 2)

The growth medium consisted of DMEM (without L-glutamine), with 4.5 g/l dextrose (ICN Pharmaceuticals ref 12-332-54) + 20% foetal bovine serum (Sigma ref :F2442, batch 51H0496) previously screened and selected to grow keratinocytes, + Glutamine 0.4 mM/ml, + Penicillin 20 units/ml and Streptomycin 20 µg/ml (Sigma ref: G6784). During incubation, the culture medium was discarded and replaced with freshly prepared medium on every third or fourth day.

Avoiding contact with the coverslip preparations, the growth medium was gently pipetted into the flasks, positioned vertically. By gently tilting each flask, the medium was allowed to fill each coverslip sandwich by capillary attraction and without trapping any air pockets. The culture flasks were incubated horizontally at 36.5°C in a water saturated atmosphere of air,

containing 6% CO<sub>2</sub>, and left undisturbed for three days to allow the epithelium outgrowth to settle, spread and adhere to the coverslip substratum.

The control epithelium, used to monitor the quality of culture media and incubator environment, consisted of slivers of epidermis (3-4 mm X 0.5mm) taken from my forearm and cultured in different flasks at the same time as the cholesteatoma. The growth characteristics of this fresh control epithelium has remained reliably consistent over a period of many years.

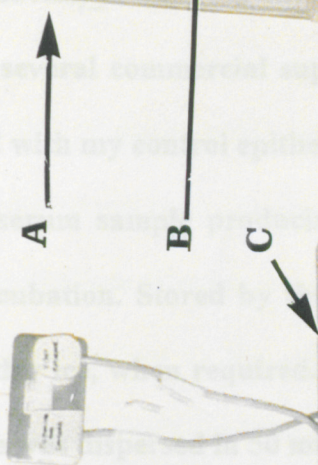
Normal tympanic membrane epithelium is always in short supply for the purposes of research, therefore some of my earlier video recordings (Boxall et al. 1988) and previously unreported observations using foetal and post mortem membranes were also used for the purposes of comparison.

#### 6.1.4 VIDEO TIME-LAPSE MICROSCOPY

Selected flasks, containing explant outgrowths of epithelium and fibroblasts, were recorded by time-lapse video using a Nikon Diaphot inverted microscope (Hoffman interference optics) housed in a perspex incubator (36.5°C). This was fed with 6% carbon dioxide, in a water saturated atmosphere, from the main incubator by using a pipe line fitted with a small pump and in-line filter to exclude bacteria. The pH of the medium, containing phenol red, could be checked visually. A Hitachi camera (VKC 150ED) was connected to a JVC video cassette recorder (BR 5925E) set to automatically registered single exposures at 3.2 second intervals on VHS tape (Fig 6.1.1).



A: PERSPEX INCUBATOR (36.5°C, 6% CO<sub>2</sub>)  
 - HOUSING AN INVERTED MICROSCOPE  
 B: CULTURE, IMAGE TRANSMITTED TO -  
 C: TIME-LAPSE VIDEO RECORDER AND  
 MONITOR SCREEN





Video recordings began on the ninth day of incubation when features peculiar to these epithelial explant outgrowths from the neck of a cholesteatoma sacs were seen to be different to cultures of tympanic membrane and explants dissected from the main body of cholesteatoma cysts.

My previous failures to sustain tissue cultures prepared from neoplastic epithelium during 1965 (unpublished) resulted from (1) using different batches of foetal calf serum and (2) allowing overgrowth of fibroblasts. Successful, long-term culture was achieved as follows:

(1) Small samples (6X50ml) of different batches of foetal calf serum in dry ice were obtained from several commercial suppliers. These were incorporated in DMEM and tested with my control epithelium. A bulk order (10 X 500 ml) was placed for the serum sample producing the best epithelial outgrowth within 3 - 4 days incubation. Stored by the supplier, single bottles (500ml) were dispatched, in dry ice, when required. In the tissue culture laboratory, aliquots of this serum was dispersed in 50 ml quantities, stored deep frozen (-20°C) and used within one month. The bulk stock, held by the supplier, was guaranteed not to deteriorate within 4 years.

My conclusion was that there were probably sufficient additional endogenous growth factors in the explants for epithelium outgrowths to become established. The presence of autologous fibroblasts and the screened foetal calf serum, was then sufficient to sustain the culture.

(2) Excessive populations of fibroblasts encroaching on epithelium were removed by scraping them off the the wall of a flask with a sterile, hockey-stick shaped glass rod, and discarding the suspended cells. These culture

flasks were rinsed with fresh changes of DMEM and replenished with growth medium.

#### 6.1.5 RESULTS; FINDINGS RELATING TO SPECIMEN C19 (neck of cholesteatoma sac)

During dissection, the presence of a sac with fragments of tissue consistently firm in texture together with loose, floating, keratin scales were present. Light microscopy revealed moderate numbers of red cells in suspension. There were no cholesterol or other crystals present in this particular specimen. (The presence of cholesterol crystals was fairly common in other specimens although, at present, no significance is attached to this finding).

Table 5 is a list of assessments, on four separate occasions of the progress of the eight cholesteatoma cultures.

In one flask, the epithelium:fibroblast ratio was optimum. The fibroblasts populated the underside surface of the top coverslip in the sandwich and epithelium populated the top surface of the bottom coverslip (Fig 6.1.2).

The time taken for the outgrowth of epithelium from cholesteatoma C19 to first appear was normal (three to four days) and the epithelium remained viable for a period of two months. I have found this to be the average, normal, life-span ( $\pm 3$  days) of epithelium cultured from tympanic membrane and cholesteatoma explants, without using trypsinisation and subculture on irradiated fibroblasts.

To simplify my reporting, I designated 'normal' findings i.e., those from the culture of normal tympanic membrane and cholesteatoma epithelium

dissected from the main body of the sac (or cyst) as 'type I' and findings from the neck of the sac as types 'II to V'.

Type (I) 'normal' *en masse* migrating epithelium from a tympanic membrane and main body of a cholesteatoma (Fig 6.1.3), not close to the *neck* of a cholesteatoma sac. *En masse* migration of the epithelium begins after ten days incubation together with the formation of a *ridge* (Figs 1.1.5, 5.1.1) of *keratinised epithelial cells*, as previously described and situated between the leading edge and cap of keratinised epithelium. The polarity of the keratinised cells in this *ridge* are parallel with the leading edge.

Types (II) - (V) from the 'neck of the sac' contain these abnormal features:

(II) Small islets of independently migrating epithelial cells were present during the first few days of incubation. These islets (Fig 6.1.4) were small fragments that had possibly become detached from the explant during dissection. Mitoses and the migration of single epithelial cells from these islets was absent. These islets ceased migration within a few days.

(III) Non-migratory islets of epithelium were also present (Fig 6.1.5) From these, single epithelial cells could be seen to migrate independently. Some of these migrating cells joined up with the epithelial outgrowth from the cholesteatoma, others migrated in random directions. All of these migrating epithelial cells avoided direct contact with fibroblasts.

(IV) Individual epithelial cells migrated away from the periphery of *en masse* migrating epithelium. Some of these, leaving its trailing edge, migrated away in directions different to the overall direction of *en masse* migration. Other epithelial cells migrating away from the leading edge, either remained

**Table 5: EIGHT CHOLESTEATOMA EXPLANTS CULTURES,  
DISSECTED FROM THE NECK OF A SAC**

<b>Date:</b>				
<b>01.12.96</b>	<b>05.12.96</b>	<b>10.1.97</b>	<b>17.1.97</b>	<b>culture</b>
<b>(=12d)</b>	<b>(=16d)</b>	<b>(=52d)</b>	<b>(=59d)</b>	<b>discarded:</b>
<b>Flask N° and findings:</b>				
<b>1. e+</b>	<b>e+f+</b>	<b>e+ f+++</b>	<b>f+++ keratin</b>	<b>22.1.97 (=64d)</b>
<b>2. e+f+</b>	<b>e+f+</b>	<b>f++</b>	<b>f++</b>	<b>22.1.97 (=64d)</b>
<b>3. e+</b>	<b>e++ cut edge</b>	<b><del>e+++</del> f+ epidermoids</b>	<b><del>e+++</del> f++</b>	<b>29.1.97 (=71d)</b>
<b>4. e+f+</b>	<b>e++ f+</b>	<b>e+++f+</b>	<b>e+++f+++</b>	<b>22.1.97 (=64d)</b>
<b>5. e+f+</b>	<b>e+++f+</b>	<b>e+++f+</b>	<b>e+++f+</b>	<b>22.1.97 (=64d)</b>
<b>6. e+</b>	<b>e+</b>	<b>e+++f+</b>	<b>*video</b>	<b>29.1.97 (=71d)</b>
<b>7. e+</b>	<b>e++</b>	<b>e+++f++</b>	<b>epidermoids</b>	<b>22.1.97 (=64d)</b>
<b>8. e+ f+ video</b>	<b>e+++f</b>	<b>e+++f alk</b>	<b>e+++f+</b>	<b>29.1.97 (=71d)</b>

**key: e+, e++, e+++, denotes the yield of epithelium outgrowth from explants i.e., +: scanty, ++: moderate and +++: profuse, and fibroblasts (f) at different intervals (d:days).**

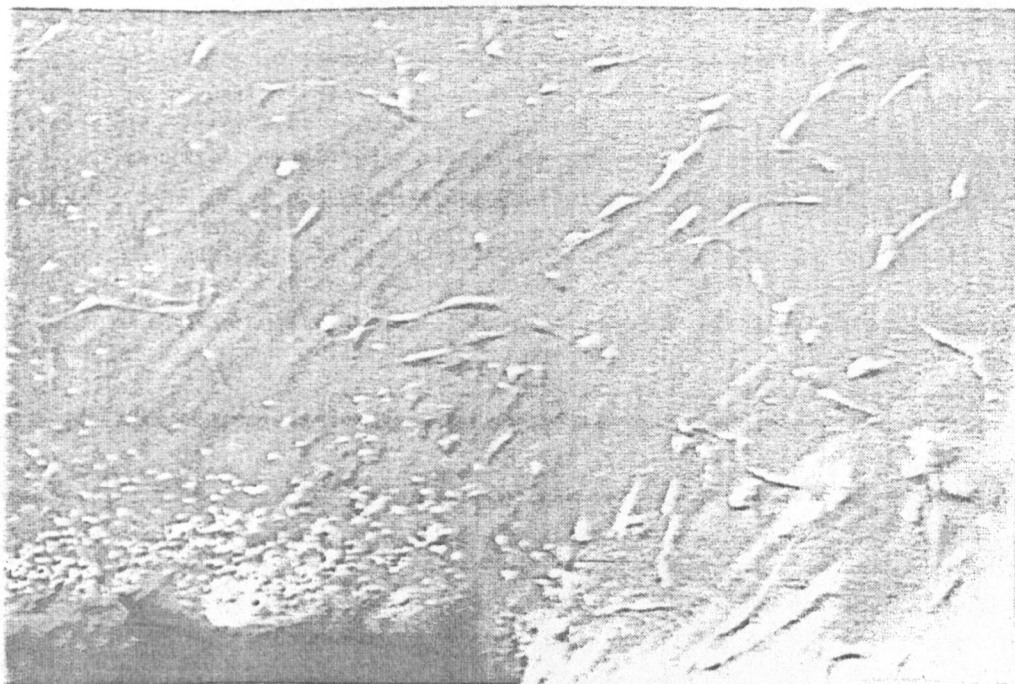
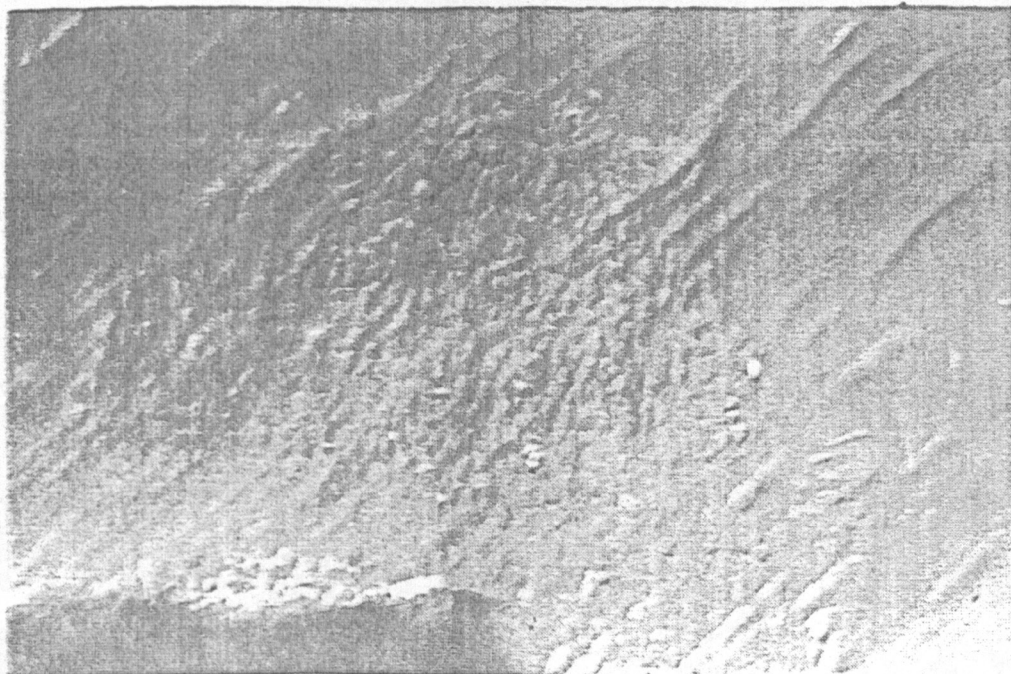
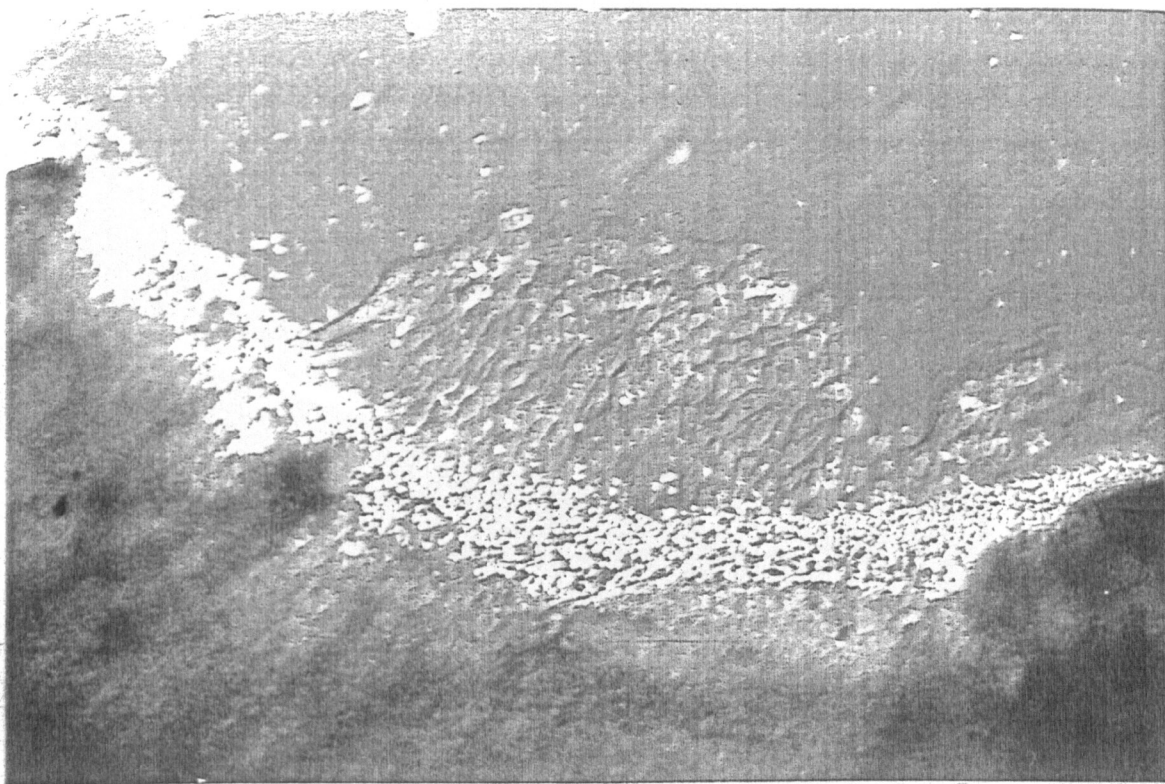


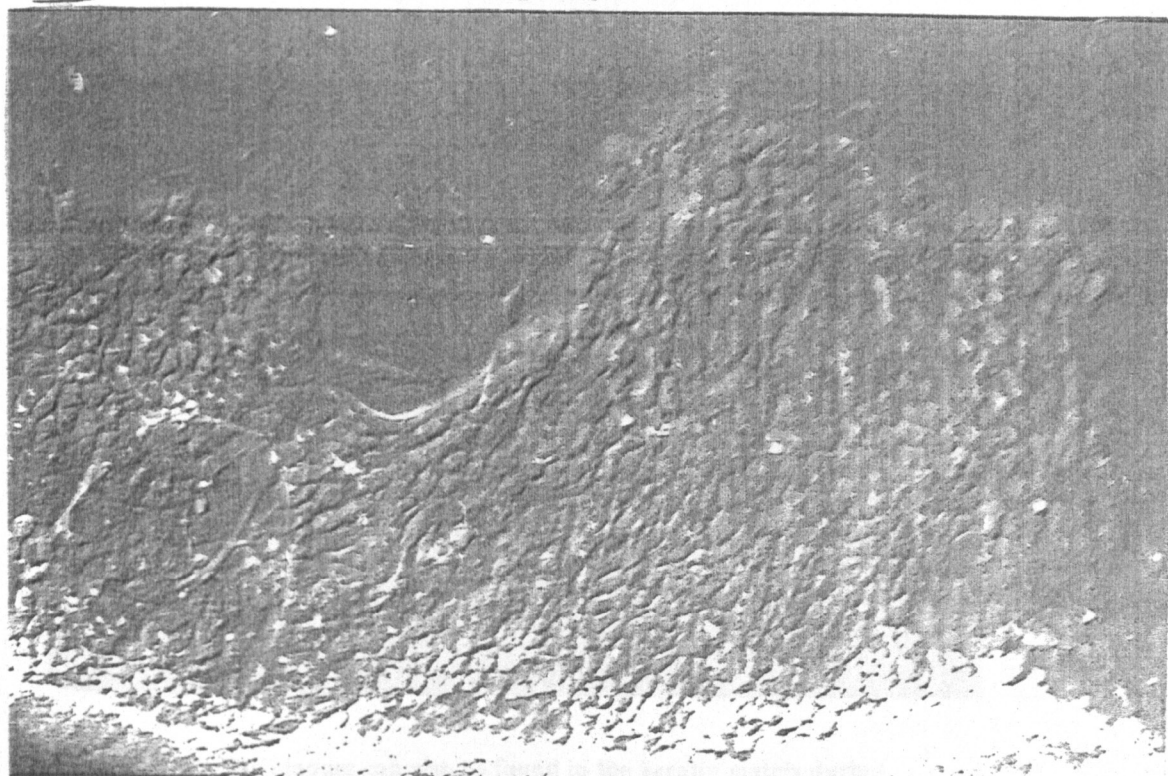
FIG: 6.1.2.

In a *sandwich coverslip* culture, the microscope is focussed (above) on the fibroblast population propagating on the underside of the top coverslip and (below) on the cholesteatoma epithelium propagating on the top surface of the bottom coverslip; an ideal arrangement for the provision of epithelial growth factor.

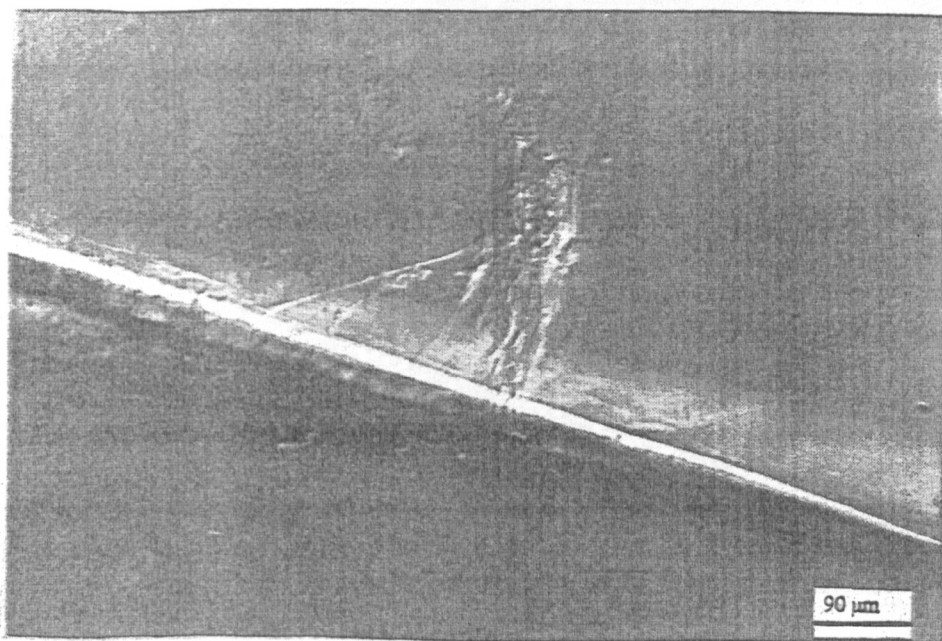




**Fig 6.1.3 Type I (see text):** Normal epithelial outgrowth from an explant following (above) 4 to 5 days incubation and (below) 7 to 8 days incubation. Note the presence of common polarity.



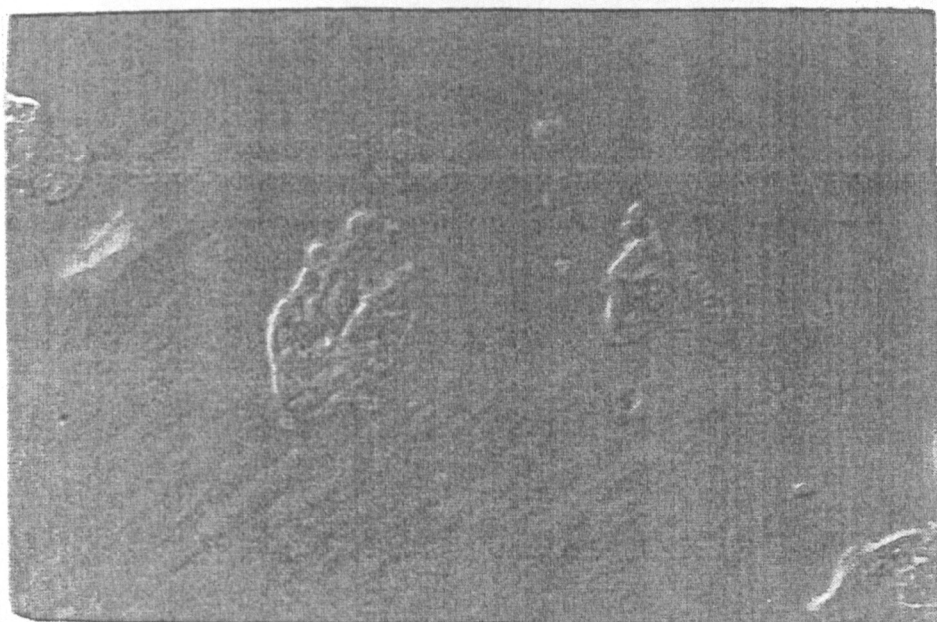




type II

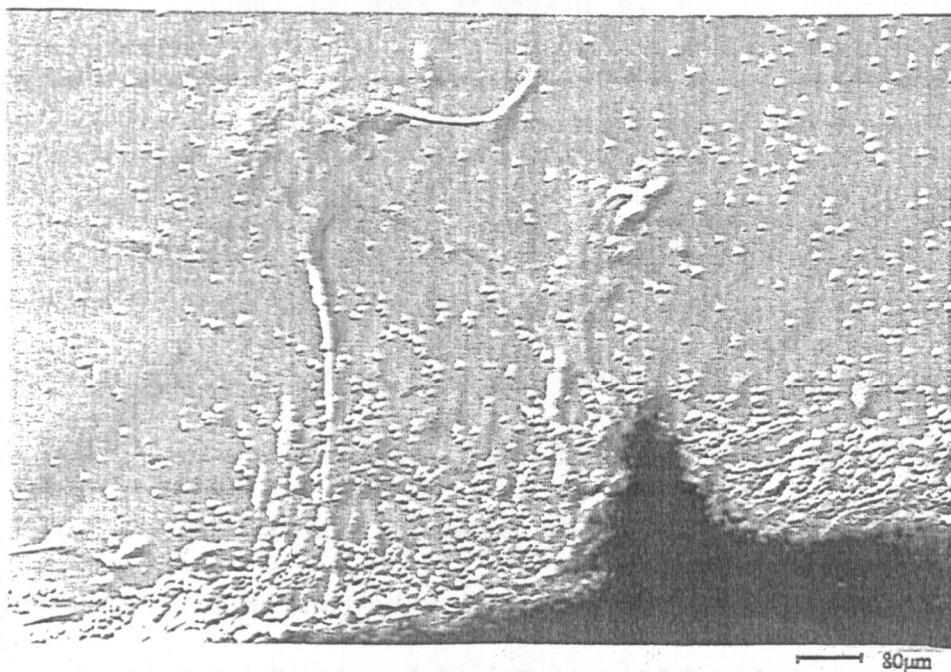
**Fig 6.1.4**

At onset and during the first few days of culture, these small, independent, migrating plaques of epithelium are found in a few cholesteatoma cultures. Usually, they rapidly deteriorate (see below and further examples overleaf).



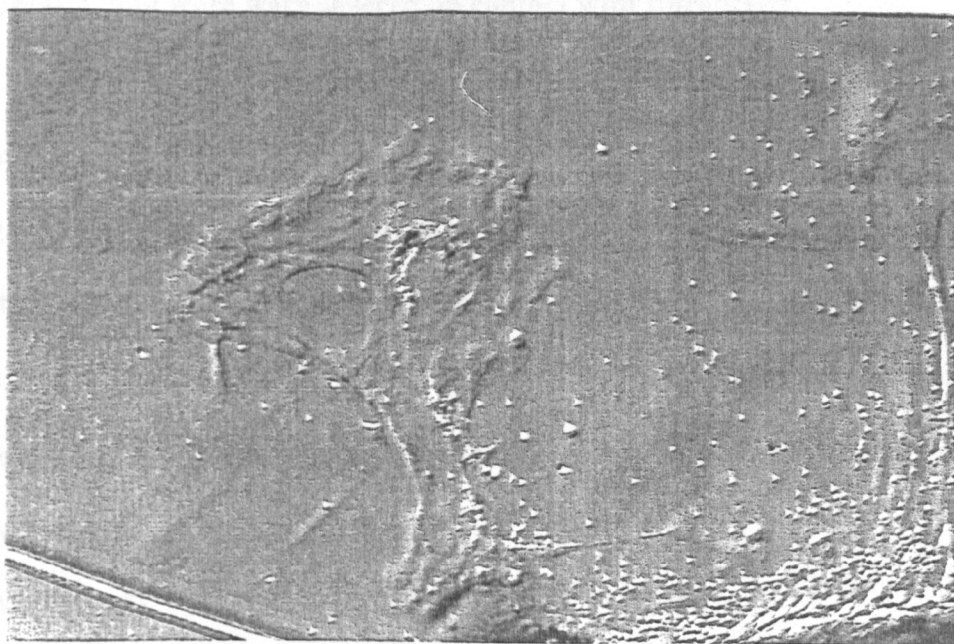
Similar plaques can also be found in the keratin matrix during dissection. Using Leishman's staining method, some of these cells appear viable, even so their culture has never proved successful.





type III

**Fig 6.1.5** Prior to en masse migration, the outgrowth of epithelium from the explant, at the bottom of the photograph, is becoming established prior to en masse migration. In addition, small independent islets are also present. Some of the epithelial cells migrating away from these islets join the outgrowth, others migrate away independently.



independent, rejoined or were overtaken by the en masse migrating epithelium (Fig 6.1.6).

(V) Individual migrating epithelial cells were seen which remained 'tethered', by a strand of cytoplasm, to the leading edge of the en masse migrating epithelium.

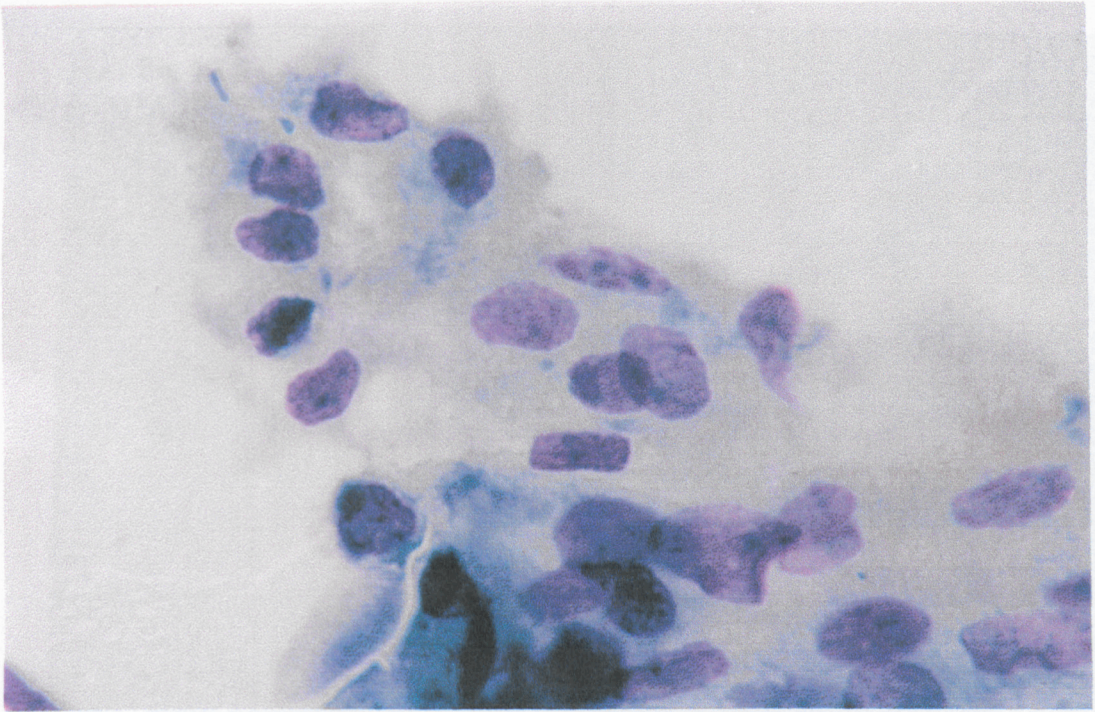
Previously, (unreported). I found similar tethered epithelium in cultures of tympanic membrane epithelium from human foetuses during the 3rd trimester. In both instances, this tether of cytoplasm formed a strong bond between the en masse migrating matrix and the migrating cell. Whereas tethered epithelial cells cultured from foetuses rejoined the matrix, some of the tethered epithelial cells in the cholesteatoma cultures anchored themselves on fresh substratum (Fig 6.1.7) and, following mitoses, produced new offshoots of tethered epithelium. These tethers, consisted of epithelial cytoplasm, and were severed on contact with fibroblasts.

In addition, the time-lapse video recordings revealed on the surface and among the top layers of the en masse migrating epithelium, small plaques of migrating epithelium. This migration resembled a '*whirling and wave-like*' motion which is best viewed by running the VHS video tape in a fast-forward mode. Unfortunately, this also produces interference lines on the monitor screen (Fig 6.1.8). Appendix 1 contains a copy of the video commentary together with duration of incubation.

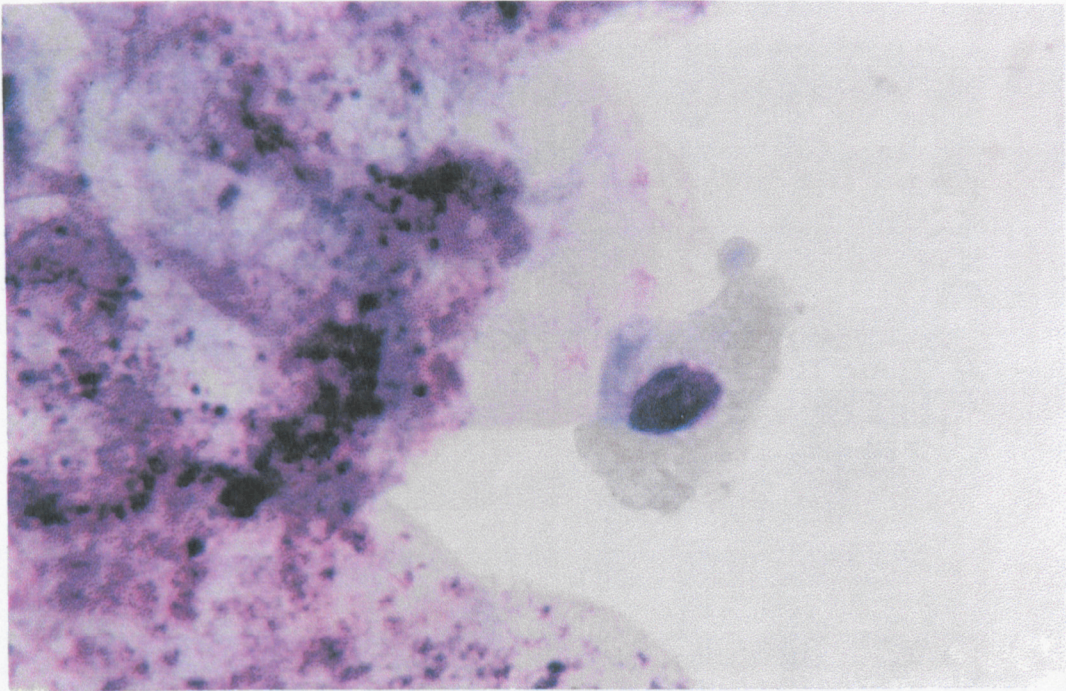
#### TIME-LAPSE VIDEO: MISCELLANEOUS FINDINGS

(i) Time-lapse video recordings of these tissue cultures revealed the destruction of epithelial cytoplasm after coming into contact with an





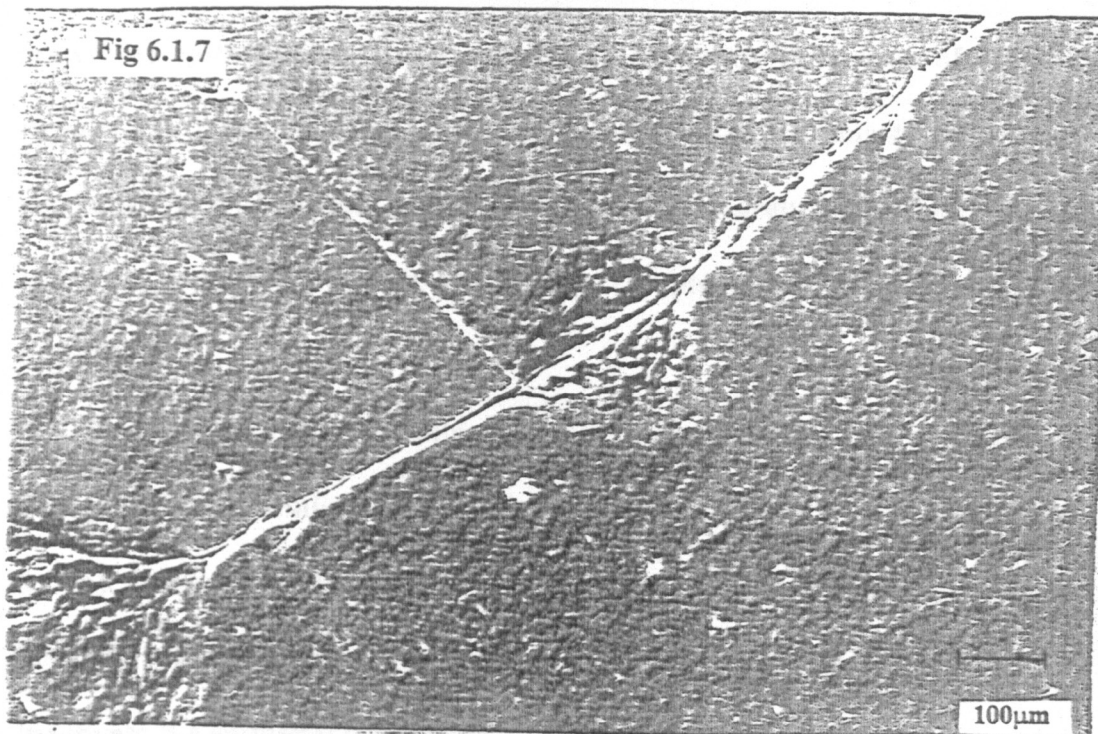
Cholesteatoma culture (fixed methanol and stained with Leishman): There are abundant healthy lamellae on this epithelium as it migrates towards the top of the screen. The cells are overlapping quite close to the edge of the culture and one cell, bottom left, is either joining or about to leave the matrix.



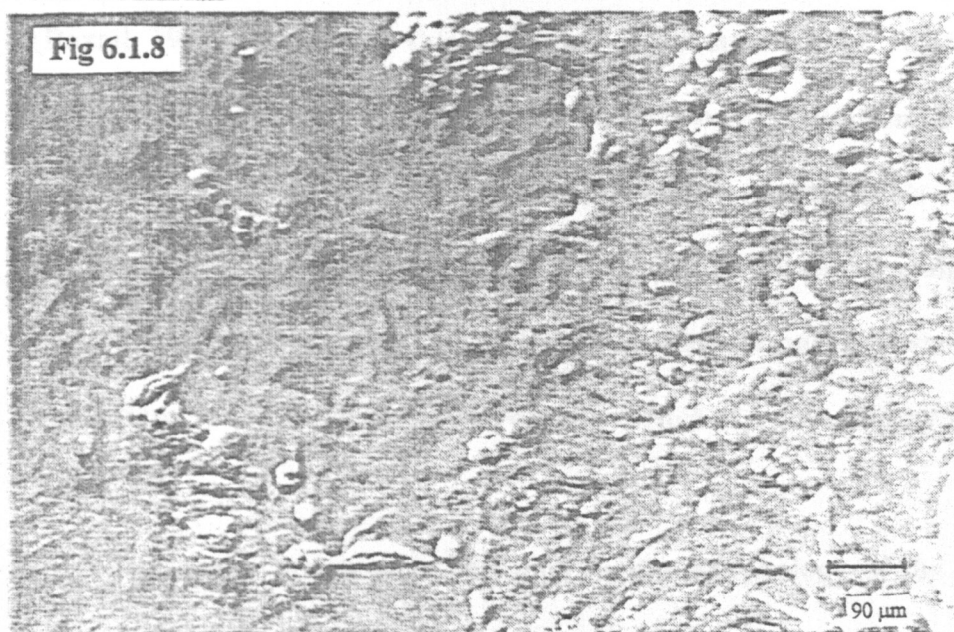
This independent epithelial cell is migrating quite close and partly beneath keratin squames. Dissected keratin from cholesteatomas can reveal small pockets of epithelium which appears viable after staining with Leishman, though culture has proved unsuccessful.

**Fig 6.1.4**





**'TETHERED EPITHELIUM':** Here, migrating epithelium remains tethered or connected to the explant outgrowth (bottom left) by a strand of cytoplasm. The offshoot of epithelium (top left) eventually rejoined the epithelial islet (centre) and the explant outgrowth eventually incorporated all of these tethered epithelial islets during its forward en masse migration.



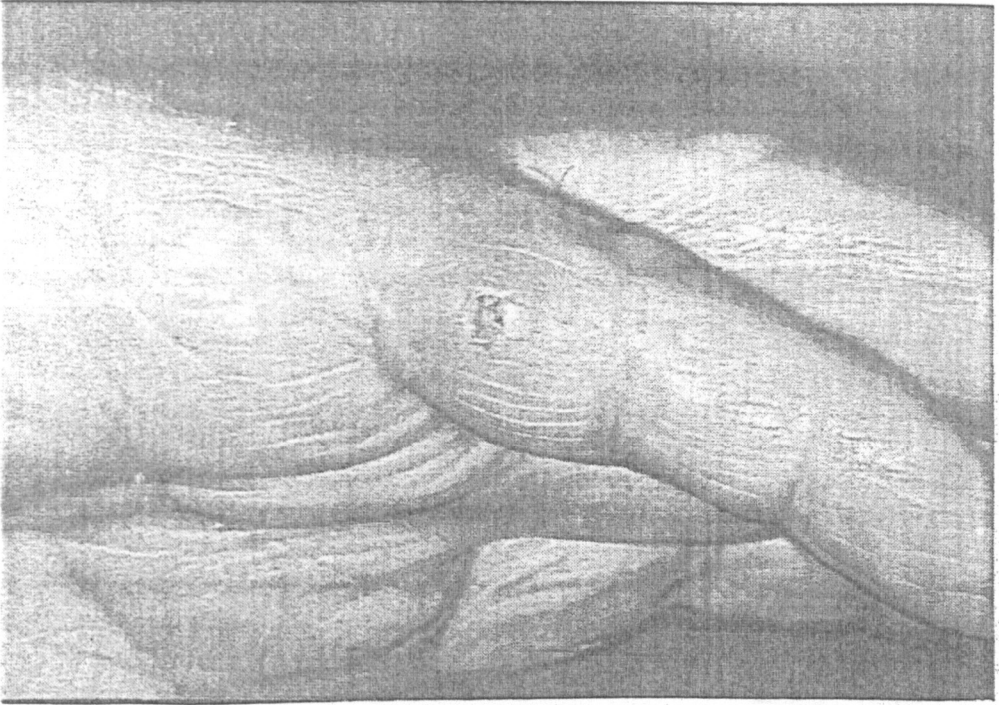
**Video.** (visible at fast speed playback and difficult to copy because this combination produces 'interference lines':) These are groups of *whirling* epithelium in the mature matrix of a few cholesteatoma cultures. Multi-layered, independent, some on the level of the surface and often migrating contrary to the direction of en masse migration in the basal layers.

unidentified cell and fibroblasts (see Appendix 2). Whereas an abundant growth of epithelium in tissue culture, aided by the protection of keratin strands can resist and even repel small encroaching fibroblast populations and cause them to retreat, single epithelium were susceptible to close contact and were destroyed by implosion of their cytoplasm following contact with fibroblasts.

(ii) *In vivo*, injury to skin which involves epithelium becoming buried beneath the epidermis can result in the formation of an epidermoid (Fig 6.1.9a). *In vitro*, small islets of migrating epithelium are often converted to non-migratory 'epidermoids' when surrounded by fibroblasts, which inactivate the epithelial lamellae (Fig 6.1.9b).

(iii) During *en masse* migration, basal epithelium in the centre of the matrix is probably protected from the harmful effect of bacterial toxins (Boxall 1993). Following this report, I have successfully cultured fresh outgrowths of epithelium after careful dissection (Fig 6.2.0) and removal of surrounding epithelium following exposure to toxins. This ability of cholesteatoma dissected from the neck of a sac to repeatedly migrate over areas of a substratum is also evident when, unlike normal *en masse* migrating epithelium, it can extricate itself from the corner of a coverslip by migration. (Fig 6.2.1).

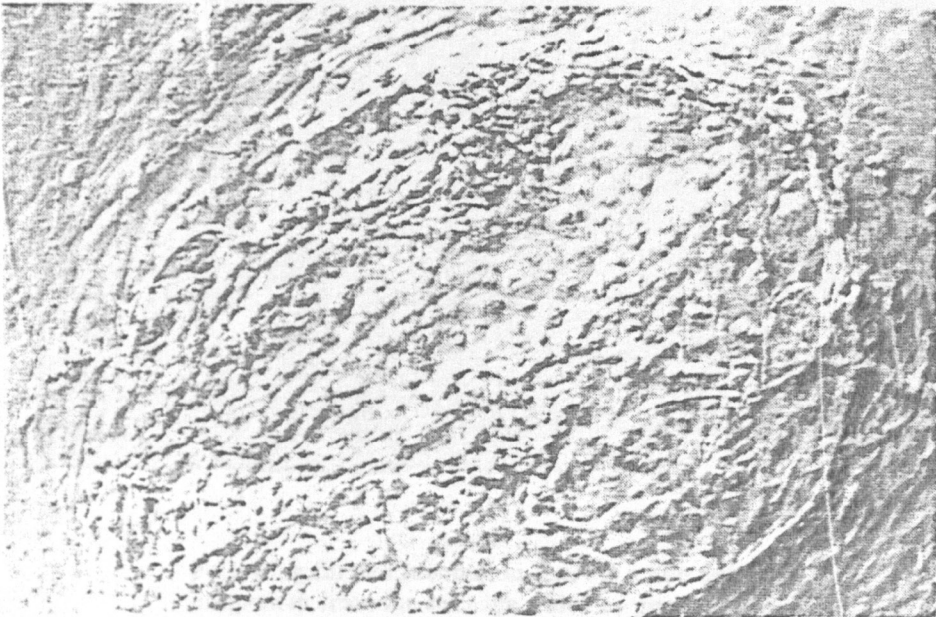
(iv) There was a wide variation in the morphology of the fibroblast population (Fig 6.2.2), some were short and squat cells, others long filamentous forms. Some crescent shaped fibroblasts were seen to glide, with



(Fig 6.1.9a)

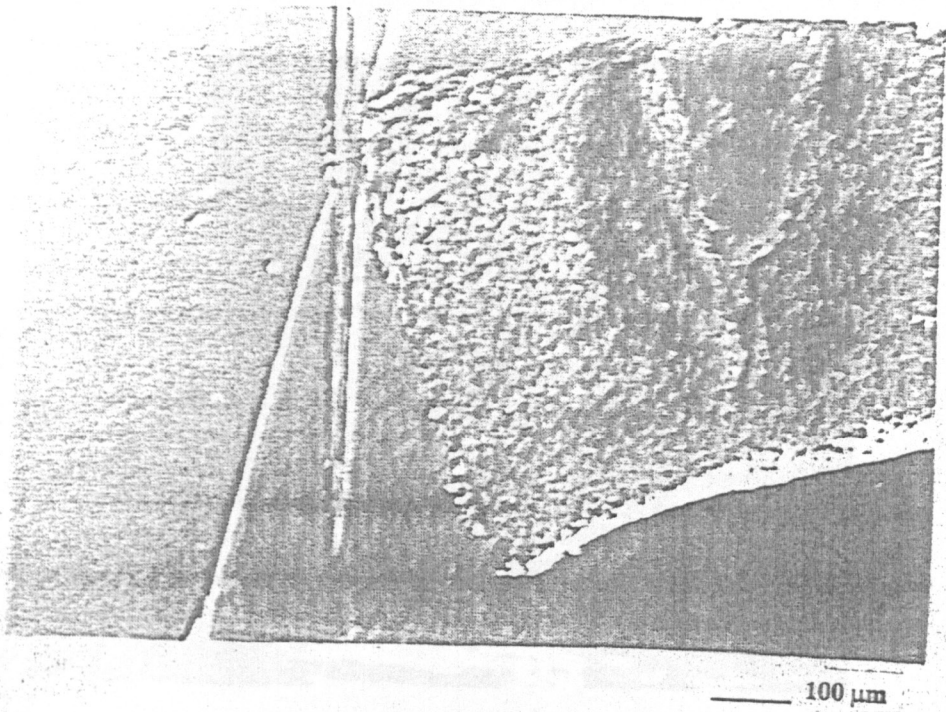
An injury to one of my fingers from the curved nail of a rabbit buried epithelium below my epidermis and produced an epidermoid which was eventually ejected.  
(below:) In this tissue culture, fibroblasts have surrounded migrating epithelium, immobilised the lamellae and produced an in vitro epidermoid.

(Fig 6.1.9b)

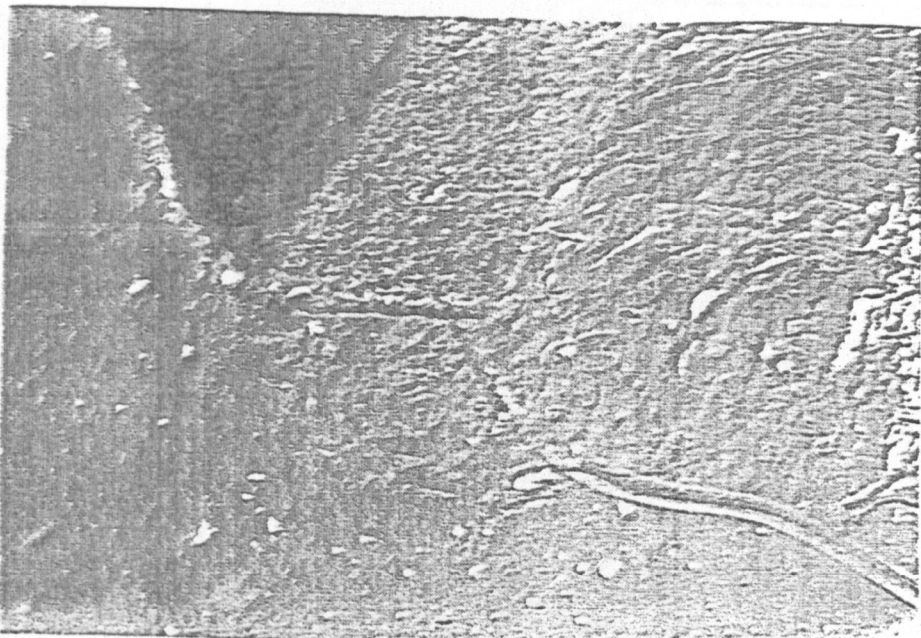


30  $\mu$ m



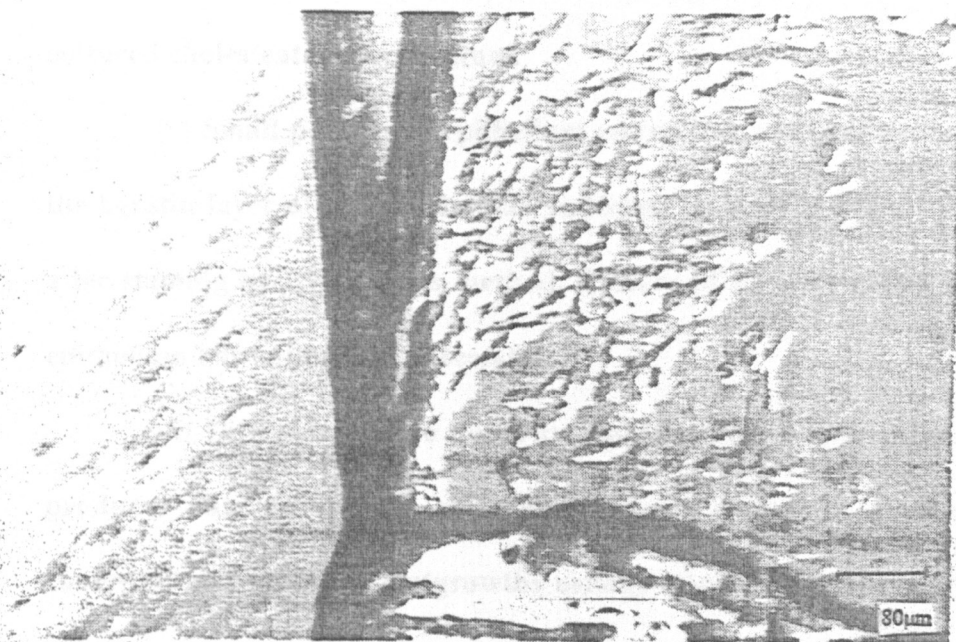


**Fig 6.2.0** The matrix of the epithelial outgrowth is cut and removed to leave a clean edge.



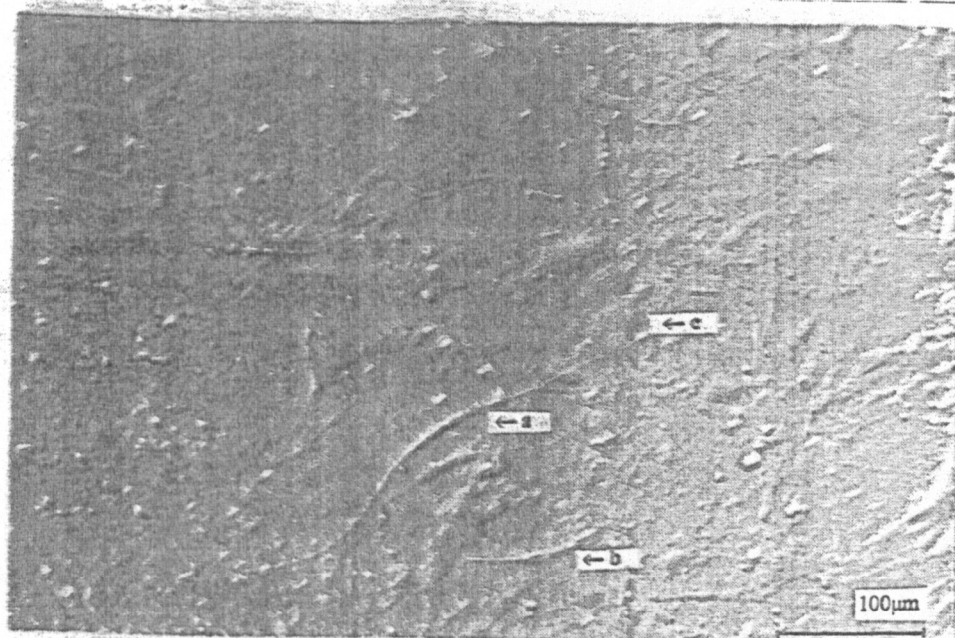
Following further incubation, there is a new epithelial outgrowth from the cut edge which re-populates the cleared areas.





**Fig 6.2.1**

(Video:) On migrating into the corner of a coverslip sandwich, tympanic membrane and most cholesteatoma epithelium mature in situ and extrude keratin squames into the surrounding medium. Here, the corner area has been repopulated by cholesteatoma epithelium (see text).



**Fig 6.2.2**

30 d incubation: This epithelial culture was sacrificed in view of the overgrowth of fibroblasts. The wide variety here includes:  
a) long slender fibroblasts, sluggish, often stationary  
b) crescent shaped, gliding migration, fast velocity  
c) short and squat, stationary

the velocity and adhesive property of lymphocytes, across the surface of the cultured cholesteatoma epithelium.

(v) Small pockets of epithelium were often revealed by teasing apart the keratin layer of cholesteatomas and some of these appeared to be viable after staining by Leishman's method. Culture of similar, unfixed pockets of epithelium has not proved successful.

(vi) Differences were noted in the duration of incubation needed to produce outgrowths of epithelium from tympanic membrane and cholesteatoma explants. Outgrowths usually appear after three or four days incubation (Fig 6.1.3). Most delays can usually be accounted for by one or more of the following reasons: the presence of too numerous erythrocytes or viscous serum which can impede the outgrowth, evaporation of moisture if the mounting procedure is interrupted and delayed, epiboly - the need for epithelial cells to migrate and cascade down the side of an explant in order to reach and spread on the coverslip substratum for propagation and the disturbance of culture flasks during the first few days of incubation.

## CHAPTER 7

### DISCUSSION

Before beginning this thesis, I cultured squamous cell epithelium of the human larynx by the methods of tissue culture (Boxall et al., 1986) followed by tongue, scalp and 'tympanic membrane epithelium', when I observed that established outgrowths from the latter migrated *en masse*. Before publication (Boxall et al., 1988) I became aware of similar work on tme, carried out by Sakai et al., (1982) and it was reassuring to find that our independent estimations of the velocity of tympanic membrane epithelium *in vitro* corresponded. The *en masse* migration was previously referred to as 'streaming' and the nearest example in microbiology was *Myxobacteria*. In biology, *Dictyostelium discoideum* amoebae aggregate to form a slug-like migration during their search for food and an optimum environment. The only common finding between these examples of *en masse* migration was my video recording of whirling plaques of epithelium and the swirling of anterior cells within slugs, recently observed by Bonner (1998).

The lifespan of my epithelium, cultured from tympanic membrane and cholesteatoma explants, was sixty (+/-3) days, after which time only dead strands of keratin and no viable epithelium remained in the tissue culture flasks. Ackerman (1977) in 'Histologic diagnosis of inflammatory

Skin Diseases' estimated the average *in vivo* process of epidermis maturation to be up to 59 days which included 28 to 45 days for maturation from germinative to cornified layer, and an additional 14 days as a cornified layer. This, together with migration *in vitro*, supports tissue culture as a useful model system for the investigation of cholesteatoma.

Whilst reviewing the literature, it became evident that there were many theories about the origins of cholesteatoma and these were frequently re-examined with renewed interest, following new and improved laboratory methods (e.g., immunocytochemistry). A lack of support from former proponents of 'metaplasia' of epithelium resulted in the demise of this as one likely origin.

Given the opinion of ENT Consultants I found that many expressed their opinions in favour of an association between the presence of suppurative middle ear infections and cholesteatoma originating from reduced pressure in the middle ear and invaginated tympanic membrane. However, contrary to this, retraction of the ear drum can be an almost permanent feature irrespective of middle ear pressure. I prepared a simple model (Appendix: 2) to estimate the effect of bacteria on gas pressures in a semi-enclosed environment i.e., to represent the middle ear during upper respiratory tract infections. The model has

severe limitations which are discussed under Directions for Future Research.

My negative findings in Chapter 3 suggest that traumatic 'disruption' of the radial layer of fibroblasts has very little permanent effect, if any, upon the normal direction of epithelial migration. However, the staining of whole tympanic membranes provided a useful method for determining differences in morphology and polarity of the cell layers.

In Chapter 4, whereas cultures of migrating tympanic membrane and cholesteatoma epithelium present a ridge of keratinised dead epithelium, cultures of epithelium prepared from the neck of cholesteatoma sacs produce cones and mounds instead of a ridge. This would allow passage of some of the keratinised epithelium and could produce the 'fragmented' cap seen in some cholestatoma cultures.

Time-lapse video recordings revealed that surface and upper layers of these caps and cone-like structures are carried forward as 'plaques' on the epithelium matrix, in the same overall direction of en masse migration, at the same moving in different directions, including rotation and whorls. The presence of adjacent groups of epithelium migrating in different directions and at different levels in the epithelial strata as a *whirling* motion is very similar to the formation described by Patel *et al.*, (1989) as the presence of *whorls*. My findings suggest that some of the layers of epithelial cells in the cone-like structures consist of

plaques which migrate in random directions. Some of these supra-basal plaques of epithelium are therefore viable, unlike the dead keratin in ridge or cap formations.

In addition, cultures of epithelium, derived from the neck region of a cholesteatoma sac, exhibited many new features.

Small islets of epithelium which only survived for a few days.

Single epithelial cells which migrate away from the *en masse* migrating epithelial matrix and may rejoining it at a later stage is a feature commonly seen in cultures of malignant epithelium (personal observation).

Epithelium which is capable of repopulating areas of a substratum, e.g., the surface of a coverslip (figs 6.2.0 and 6.21).

Whereas many of these features appear minor in significance, collectively they all point towards an activity among epithelium in the neck region of a cholesteatoma sac.

The next important observation was that whilst epithelial cells could migrate around an "internal corner" they are quite unable to migrate over an edge. They may be pushed over an edge by the force of the migration of the epithelium behind this leading edge but in general epithelium tends to accumulate in this situation in vivo (Fig 1.2.6).

The reason for this apparent inability to cross an edge may be explained by the microanatomy of the epithelial cells at the limits of a



culture. The leading edge is clearly upturned in these particular cells so that adhesion between the cell and its substrate is lost and it is this that would appear to restrict the further migration over an edge. This is clearly shown in two photographs of isolated tympanic membrane epithelium climbing round the internal 90° angle of a flask (Fig 7.1.1).

We thus have an *in vitro* model that adequately explains the clinical formation of cholesteatomas whether they are arising in the pars flaccida or in an atelectatic pars tensa.

A negative middle ear pressure, perhaps caused and sustained by *Pseudomonas aeruginosa*, could result in retraction so that the membrane becomes draped over the bony rim of the deep ear canal. Initially, migration is possible around a gently curved edge but with further retraction this migration fails and the keratin accumulates at the margin. This in turn prevents further migration so that the keratin fills the retraction pocket and a cholestatoma is formed. This mechanism, suggested by my *in vitro* studies, strongly supports the *in vivo* mechanism suggested by Sade (1993b) amongst others.

An entirely different feature (Appendix:1) is the destruction of small populations of non-migrating and en mass migrating epithelium by fibroblasts. These surround and immobilize the epithelial lamellae and convert them to lifeless epidermoid formations. An unidentified killer-cell, in the video recording, first shared a compatible and possibly

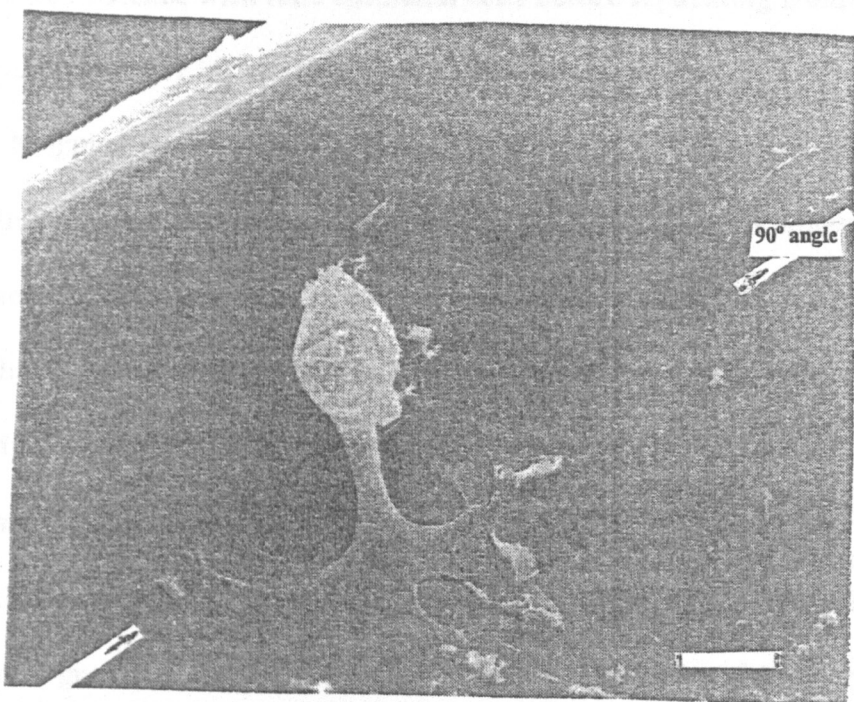
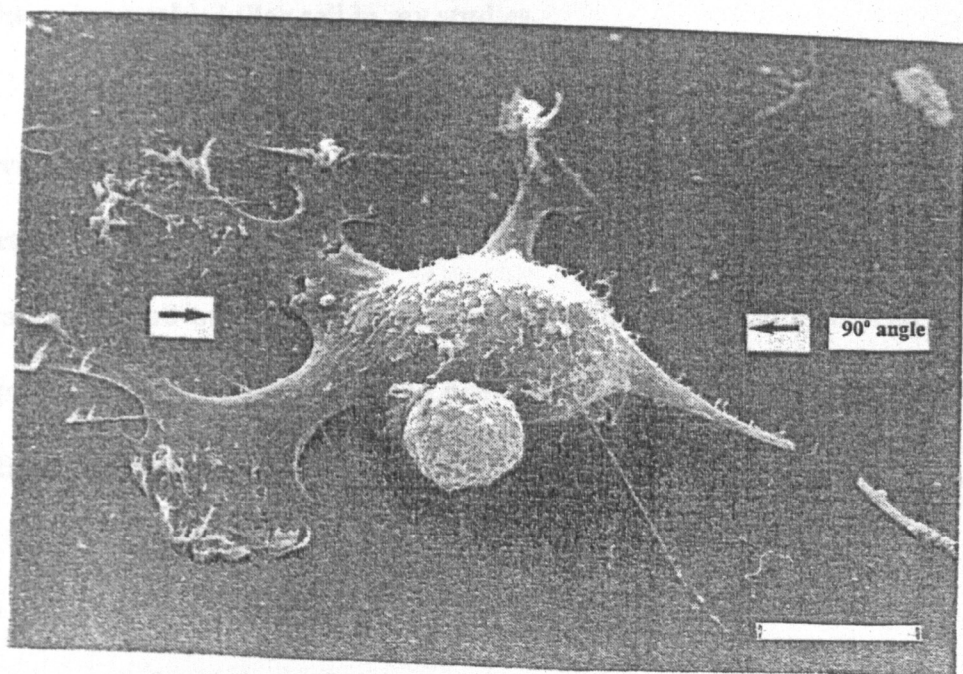


FIG 7.1.1

Tympanic membrane epithelium migrating through an internal angle of 90° to reach the wall of a culture flask  
[bar = 10 $\mu$ ]



synergistic existence with lone epithelial cells before separating from and destroying the latter by implosion of its cytoplasm.

Morphologically, the killer-cell was at first identical to an epithelial cell and there was no contact inhibition between the killer cell and the epithelial cell. There was also no contact inhibition with the fibroblasts which followed in its trail. These fibroblasts destroyed individual epithelial cells towards the end of their culture life by touching the epithelial cells with their proboscis.

During the 1992 Cholesteatoma and Mastoid Surgery Conference in Japan, great emphasis was placed upon the presence of Langerhan Cells, lymphocytes and macrophages in tympanic membrane culture. Morphologically, none of these cells, all of which are present in cultures of tympanic membrane and cholesteatoma epithelium, fit the description of the polymorphic killer cell in my studies.

It would appear that fibroblasts have at least two roles. They provide epithelial growth factor and, at least in vitro, they can destroy or inactivate epithelial cells. Bonding between epithelial cells was absent towards the end of their life span and individual epithelial cells which migrated away from their covering protection of keratin were destroyed by the killer-cell or fibroblasts.

**It is possible that the function of the scanty fibroblasts, seen in the layer beneath the epithelial basal membrane (Chapter 3), is to destroy any epithelium which penetrate their basal membrane.**

**Some workers have suggested that direct invasion of squamous epithelium through to middle layers of the tympanic membrane pars flaccida or pars tensa could be a mechanism for the formation of cholesteatoma. My results strongly suggest that this is very unlikely because of the presence of at least some fibroblasts in all cholesteatoma cultures which act to erradicate aberrant epithelial cells. This has been clearly shown in the video recording.**

**The frequent retraction of some tympanic membranes in spite of adequate ventilation and equilibrium of gases with atmospheric pressure, has yet to be investigated and satisfactorily explained.**

## DIRECTIONS FOR FUTURE RESEARCH

The model that I constructed to evaluate the effect of bacteria on pressure changes in the middle ear proved to be a useful exercise by providing some of the reductions in pressure obtained by various bacteria cultured in my defibrinated blood. There are probably few, if any refinements that can be made to the existing model and it has been suggested (personal communication) that a sealed temporal bone could be used as a basis for a new, improved design. My preliminary tests were limited to single strains of bacteria and used a fixed length of gas permeable tubing. Any gas absorption experiment needs to be repeated using gas mixtures which contain a range of gas proportions equivalent to those normally present in the middle ear. Chronic suppurative otitis media infections and cholesteatomas could be screened for bacteria and the Public Health Laboratory asked to type strains of *Pseudomonas aeruginosa*.

To determine the optimum time to fix and stain 'killer cells' for the purposes of identification could present a difficulty. Lymphocytes present a problem because of their fast erratic butterfly-like migration over, under and between epithelium, even using brief fixation techniques. Killer cells present an entirely different problem in that, during culture, they are often morphologically identical to epithelial cells.

It is very unfortunate that continuation of human foetal work is difficult because a change in law requires permission from the mother. This could have a very traumatic effect on her. The provision of tympanic membranes from fresh cadavers also presents problems.

The examination of diseased and normal human temporal bones continues to reveal the presence of many undiagnosed cases of cholesteatoma. Some of these may have contributed to the death of patients and others which probably resulted in their severe disability prior to death. Deafness among many of the elderly is often accepted as normal and if cholesteatoma is the cause, then this deafness may be preferable to major surgery. Those patients who are diagnosed and accept advice for surgery, often find that the recurrence of cholesteatoma can be an unexpected outcome.

In vivo, the prevention and destruction of cholesteatoma has been a primary object of ENT surgeons and research workers, for more than a century. At the present time, removal of cholesteatoma tissue by surgery or suction continues to be the only course of action.

Tissue culture may eventually help to find an alternative form of treatment to surgery. As discussed, the origin of many cholesteatomas is the accumulation of normal tme which eventually promotes a sac formation in the pars flaccida. This sac collects epithelium, keratin debris, cerumen, moisture and probably bacteria and fungi. As the sac



expands, the internal surface area increases and contact inhibition of basal epithelium diminishes. This, in turn, promotes propagation and, possibly, further migration of the epithelium.

I have used colchicine for arresting mitoses, in order to study the distribution of propagation during the en masse migration of epithelium (Boxall 1993). However, it is unlikely that any form of colchicine would be safe for clinical use as a topical application to arrest migration. At the same time, I also tested the effect of non-specific bacterial toxins on epithelium. This brought about cessation of migration by immobilising the leading epithelial cells. In a subsequent, unreported trial, en masse migrating epithelium cultured from the neck of a cholesteatoma sac, which had been immobilised by treatment with bacterial toxin, were reincubated after the leading edge of epithelium had been removed by gently scraping it away using the back edge of a sterile No 11 scalpel blade. The cut edge of the remaining epithelial matrix produced a fresh outgrowth of epithelium (Fig 6.2.0). This feature again illustrated the ability of epithelium from a cholesteatoma sac to repopulate an area of coverslip substratum previously colonised, and suggested that further consideration might be given to testing *Pseudomonas aeruginosa* toxin for topical application on cholesteatoma pockets.

## **APPENDIX: 1**

### **TIME-LAPSE VIDEO RECORDING (ATTACHED TO THIS THESIS)**

VHS Tape, running time approx. 15 minutes

The images in the first part (A1.1) are slightly lacking in clarity because the illumination needed to pass through two layers of plastic: the wall of the culture flask and the coverslip on which the tissue explant was cultured. In the second part (A2.1), the clarity improves because there was no coverslip to impede the light.

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#### **A1.1 PART 1: CULTURE OF CHOLESTEATOMA EPITHELIUM DISSECTED FROM THE NECK OF A CHOLESTEATOMA SAC**

Date, period of incubation

28.11.96., 9d:

This is an edited version of a time-lapse video recording of epithelial outgrowths from explants dissected from the neck of a cholesteatoma sac, and cultured over a period of sixty days. None of the features (II-VI, Chapter 6) was present in cultures of tympanic membrane epithelium or cholesteatoma cultures other than those dissected from the neck of a cholesteatoma sac.

Independent islets of migrating epithelium were seen during the first few days of culture.

Epithelial cells around the circumference of this islet are pulling it in two directions i.e., epithelial cells are tethered by a strand of cytoplasm to

the islet in a manner previously seen during the culture of foetal tympanic membrane epithelium and described elsewhere in this thesis. Eventually, the cytoplasm of the epithelial cells on the top right-hand fork is stretched to breaking point and when the cells are free, they migrate away from the islet to join the outgrowth of the cholesteatoma explant epithelium situated just off the right-hand side of the screen. Meanwhile, motile lymphocytes migrate in a typical, random butterfly fashion among the non-motile lymphocytes and other cells e.g., erythrocytes and leucocytes.

The left-hand fork also breaks free, one branch of this epithelium migrating away independently to the left and the other branch again migrating to eventually join the outgrowth of explant epithelium. Yet another branch on the right hand side of the islet attaches itself to the outgrowth and the islet itself is pulled along with it towards the epithelial outgrowth.

On the tenth day of culture, an epithelial cell on the bottom edge of the islet can be seen to migrate away and then reverses direction, just before disappearing off the bottom of the monitor screen. It is therefore possible to follow its progression in the new and opposite direction.

Its progression is fairly linear, as is that of a similar epithelial cell heading down the screen, in the opposite direction. The two cells meet, there is no contact inhibition and they merge together and briefly gyrate, before parting company and heading back in the direction they came from.

29.11.96., 10d: The second migrating epithelial cell now approaches a horizontal, elongated fibroblast and, this time, there is inhibition before any form of contact is made, and the epithelial cell seeks a way to evade the fibroblast by passing either side of it. Part of the epithelial cell cytoplasm becomes detached and migrates away. This event is not uncommon; the remnant of cytoplasm will briefly migrate and then disintegrate. The fibroblast has contracted, and the epithelial cell eventually manages to join the epithelial outgrowth.

Individual confrontations in 'young' cultures usually result in the survival of epithelium. In mature cultures fibroblasts are less likely to contract in the above manner. Confrontations between large populations of fibroblast and smaller populations of epithelium usually result in the fibroblasts surrounding the epithelium, restricting movement of their lamellae and converting them to what may be described as in-vitro 'epidermoids'.

07.12.96., 18d: Outgrowth of a sheet of epithelium from the cholesteatoma explant now populates the right hand side of the microscope field and it gradually pushes a strand of keratin against the fibroblasts which occupy the left hand side of the field.

This is the centre of the matrix where motile lymphocytes continue to climb over, burrow under, and migrate between the epithelium. The problem with identifying individual lymphocytes using staining methods e.g., immunocytochemistry is that during fixation they can be hidden under the

epithelium and are also indistinguishable from the large population of non-motile lymphocytes present in cultures but only positively identified when the tissue is stained.

At the tail end of the en masse migrating matrix, each epithelial cell is stretched as it is pulled forward, and its surface area can exceed that of the leading epithelia, fivefold. One of the epithelial cells at the tail end of the matrix migrates in the opposite direction to overall en masse migration, which is from left to right.

Close to the leading edge, epithelial cells leave, and sometimes rejoin the migrating matrix. There is a considerable difference in the size of the epithelial cells and some at the top right hand corner exceed 100 $\mu$ m in diameter.

#### DISCUSSION

In the first part, this tissue culture model suggests that some of the squamous keratinising epithelium, from the surface of the tympanic membrane, which gains entry to the middle ear by way of invagination of the drum, undergoes a temporary transition in its morphology and migratory habits which enable it to gain a foothold in the middle ear. *In vitro*, the overall velocity of en masse migration and its linearity, which is dependent on the basal layer, was not affected by the superficial sheets and plaques of whirling epithelium.

By viewing the video recording in a fast-forward mode, some of the epithelium can be seen to migrate in a whirling motion which is different

to the direction of en masse migration. Unfortunately, at the same time, by increasing the speed of tape recordings, interference lines are produced on the video screen. Fast forward viewing shows whirling epithelium where migration does not necessarily follow the same direction as that of en masse migration. Patel et al., (1989) when observing cholesteatoma sacs, stained with haematoxylin and eosin and flattened between glass sides, reported the presence of 'whorl' patterns which suggested disorder or frustration of normal migration, show no single direction of alignment, producing frustration of normal direction. All of these descriptions were found in cholesteatoma explants dissected from the neck region of cholesteatoma sacs in the current series for this thesis. These explants also produced both a high yield of epithelium and the presence of epithelial cell types II - VI.



**A1.2: *IN VITRO* DESTRUCTION OF EPITHELIUM FOLLOWING CONTACT WITH AN UNIDENTIFIED CELL AND FIBROBLASTS**

**Date, period of incubation**

**27.12.96., 38d: (Fig A1.2.1) Four epithelial cells are exposed at the edge of this keratin matrix, under which they migrate as some form of protection in the presence of fibroblasts. Occasionally, these independent epithelial cells are barred from retreating in this manner by intervening fibroblasts.**

**29.12.96., 40d: Here, one epithelial cell is quite independent and another cell, with similar morphology, enters the field of view at the bottom right hand corner of the screen (Figs A.1.2.2-8) Without migrating away, the epithelial cell briefly retracts its cytoplasm as the new cell advances, and at the same time the new cell changes its shape and the intensity of its nucleus. It is possible that the epithelial cell senses the presence of epithelial growth or some other beneficial factor as, eventually, the new cell manages to initiate close contact. Considerable changes in the morphology of the new cell continue to take place. While this happens, there is time to explain the means by which the clarity of this image came to be improved.**

**Migrating tympanic membrane or cholesteatoma epithelium grown in liquid culture medium cannot grow across a sharp edge and down or over the side of a support material e.g., over the edge of a coverslip.**

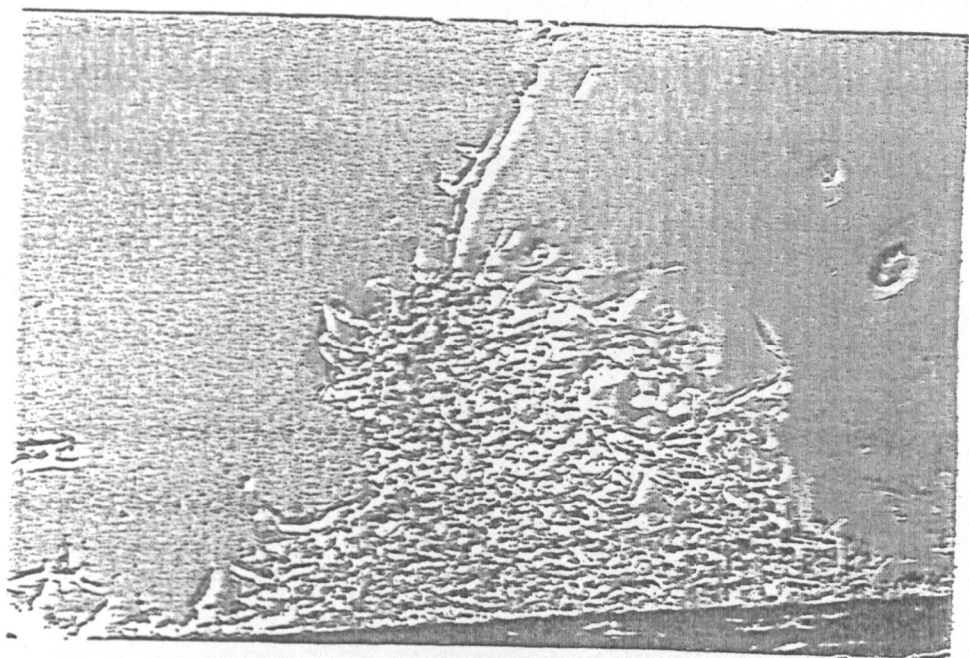


Fig A1.2.1

— 20 $\mu$ m

Two epithelium have migrated away from the matrix, others partly emerge from the keratin matrix, under which they will retreat if threatened by the presence of fibroblasts

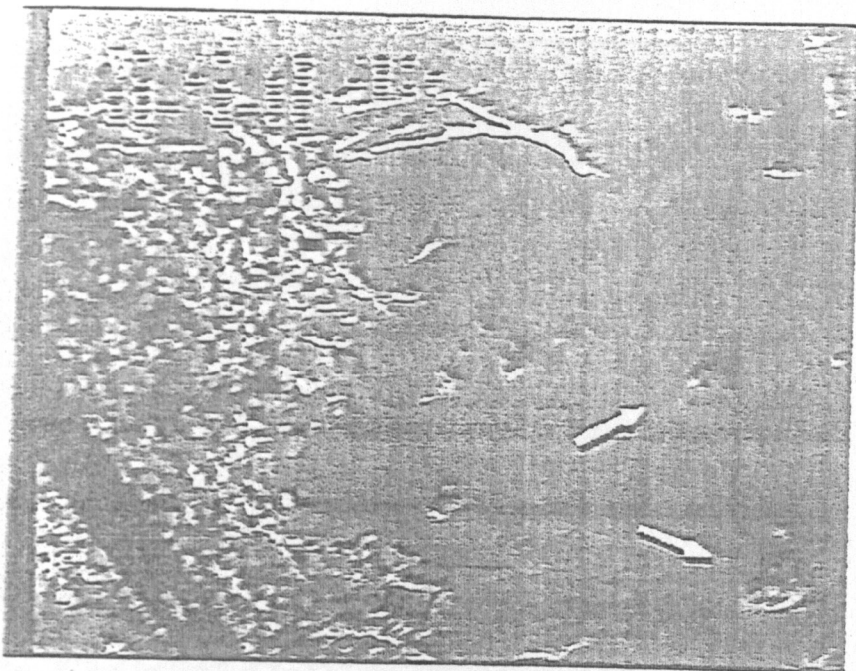


Fig A1.2.2

29.12.96 10.40h: The edge of the the coverslip sandwich can be seen in the bottom left hand corner together with extruded keratin. An isolated epithelial cell (top arrow) is being approached by what appears to be a second migrating epithelial cell (bottom arrow).

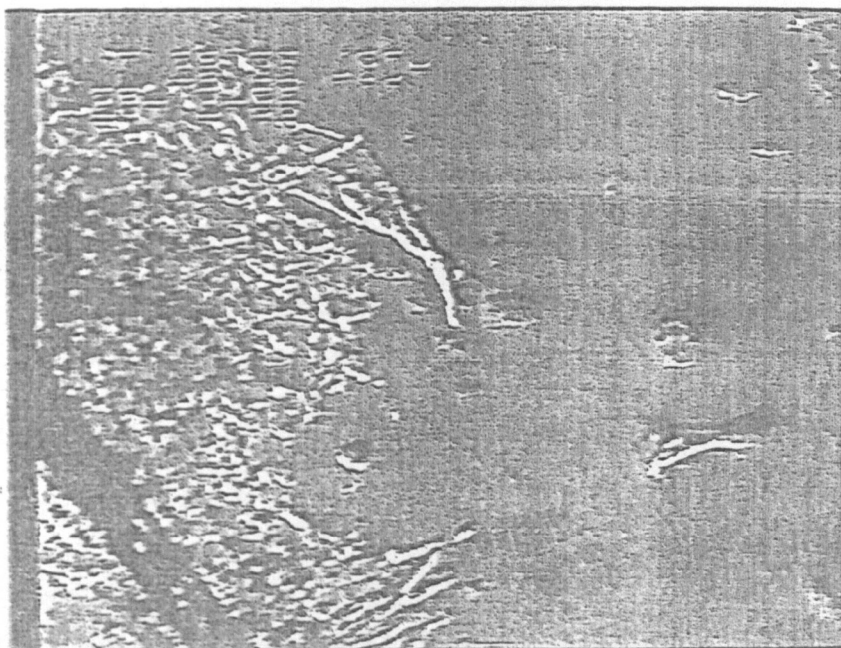


Fig A1.2.3

29.12.96 17.29h: The morphology of the second cell has changed from round in shape to elongated with polar lamellae and eccentric nucleus facing the keratin matrix.



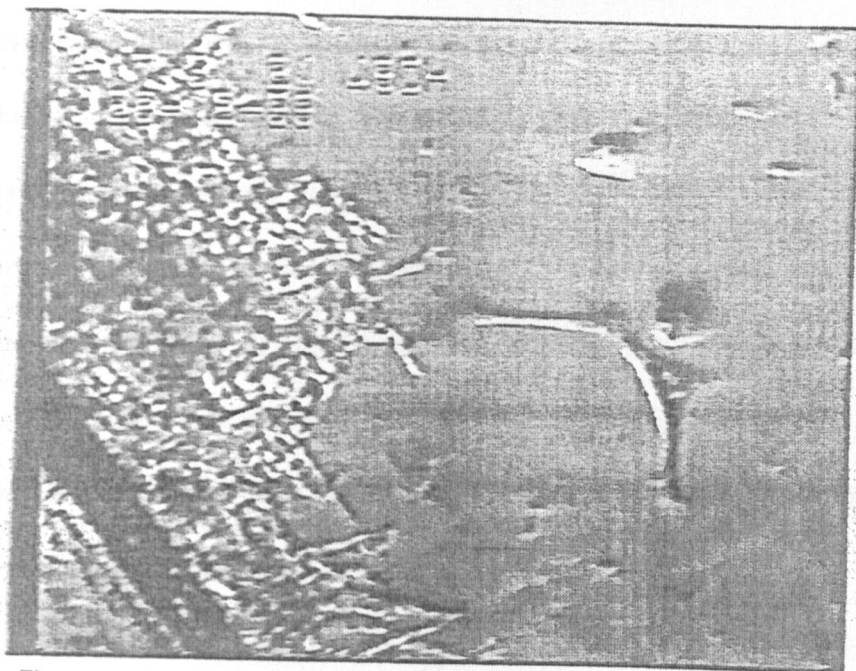


Fig A1.2.4

29.12.96 17.31: The lamellae is equally attracted to epithelium beneath the keratin matrix and trailing fibroblasts. In addition, it partly encircles (1/3rd) of the epithelial cell and both cells appear to share a common cytoplasm.

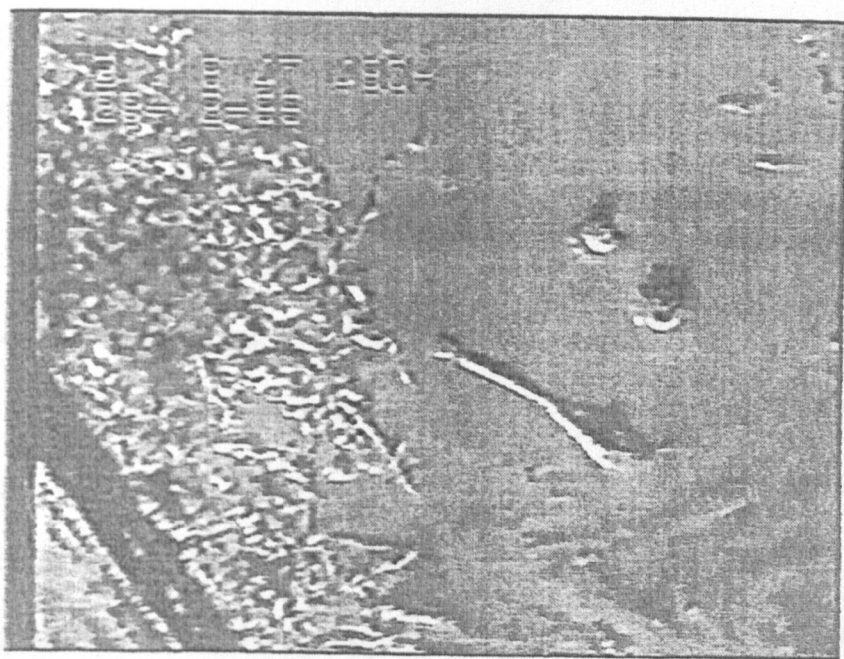


FIG 8.2.5

29.12.96 21.13: The intruding cell remains attracted to epithelium beneath the keratin matrix and trailing fibroblasts after separating and migrating away from the epithelial cell. The cytoplasm of the latter will suddenly implode, without first blebbing, to leave behind a dead nucleus.

However, it can grow along the base and up the side of a flask wall. I believe that this occurs because of lamellae on the apical surface of epithelial cells and leading upturned proboscis. Conversely, when a migrating epithelial cell reaches the vertical precipice at the edge of a coverglass substratum, it can only turn left or right. On reaching the corner of a coverslip, it cannot turn through  $90^{\circ}$  or backtrack, instead it stops migrating and the cells mature in situ and the resulting keratin strands are extruded into the liquid culture medium and carried away by convection currents as they break away..

Here, on the left of *the screen*, the weight of accumulated keratin which happened not to disperse, pressed the epithelium down on to the wall of the flask and the image is only transmitted through a single thickness of plastic i.e., the flask wall.

29.12.96, 40d: Fibroblasts are now entering the field in the wake of the new cell.

The cytoplasm of the epithelial cell and the new cell now appears to be continuous. Although the new cell has been attracted towards a second independent, blebbing, epithelial cell, since the beginning of this sequence, this second cell has now moved away. The new cell is now triangular in shape with three, fan-shaped, corner lamellae directed towards the epithelial cell, trailing fibroblasts and epithelial cells which have retreated beneath the protective keratin matrix. It begins to probe the latter,

perhaps attracted chemotactically to the underlying epithelium but it is also contact sensitive to the strands of keratin.

The new cell has disengaged itself from the epithelial cell which appears to be unharmed. However, shortly afterwards, it suffers a sudden violent implosion of cytoplasm, leaving behind an inert nucleus.

Perhaps it could be justified to refer to the new cell as a "killer cell" as it will now proceed to migrate along the edge of the keratin matrix.

31.12.96., 42d: Epithelial cells in other parts of the culture are isolated from the keratin matrix by fibroblasts, of which there is a wide variety.

17.1.97., 59d: Some of these fibroblasts are bird shaped in profile, others are more typical in shape and yet others are spindle shaped. The typical fibroblasts, seen here, are having the same devastating effect upon the epithelial cells as the killer cell in the previous sequence.

#### 8.2.2 DISCUSSION

In the second part, it appears that some fibroblasts play a significant role in destroying epithelial cells which migrate through breaches in the epithelial basement membrane resulting from infection. These fibroblasts could possibly be sensitised and triggered to kill epithelial cells by the unidentified killer cells. It is also possible that these fibroblasts occupy the sparsely populated connective tissue (Experiment 2) adjacent to the epithelial basement membrane.

Normally, epithelium lining the middle ear does not come into direct contact with mastoid bone; the two are separated by a membrane.



Similarly, keratinising, stratified, squamous epithelium does not come into contact with fibroblasts. From the video results, one could suppose that relatively few potential cholesteatomas ever reach fruition.

Following wounding, fibroblasts responsible for the repair of human dermis come from the subdermal tissue. Fibroblasts in the dermal layer of the connective tissue remain inactive due to inhibitory substances (Gillman et al., 1955). Muir et al., (1977) found that whereas extracts of the extracellular matrix of the dermis made with citric acid/citrate buffer pH 3.5 caused inhibition of fibroblast growth, no obvious differences existed between the inhibitory activity of the fractions of mature scar tissue extracts as compared with extracts of normal dermis. This suggests that there is a permanent limiting inhibitor of fibroblasts growth in the dermis.

## **APPENDIX 2:**

### **A MODEL TO TEST THE EFFECT OF BACTERIAL INFECTION ON MIDDLE EAR GAS PRESSURES**

#### **A2.1 INTRODUCTION**

In a simple survey involving 45 ENT consultants, I found that 27 (60%) emphasised, in their opinion, a connection between infection and cholesteatoma. I therefore prepared a simple model to estimate whether micro-organisms might produce sufficient reductions or fluctuations in middle ear gas pressures to produce retraction pockets in the *pars flaccida*.

Middle ear pressure and mastoid pneumatization appear to be interrelated, the latter possibly acting as a pressure buffer to the middle ear space (Sade 1992). The size of mastoid pneumatization probably affects the development of retraction pockets which are secondary to fluctuating negative pressure in the middle ear. Blockage of the tympanic isthmus, which connects the mesotympanic portion of the middle ear and epitympanum, may affect the aeration of the temporal bone pneumatic system and play an important role in the pathogenesis of cholesteatoma (Yoon *et al.*, 1993).

Transmitted pressure is a significant factor in the activation of osteoclasts and stimulation of osteoclastic bone resorption in the middle ear of gerbils (Wolfman & Chole 1986) and the pressure produced by expanding aural cholesteatomas has been implicated as a causal factor in the induction of osteoclastic resorption of adjoining bone.

Abramson (1989) found that reduced pressure in the middle ear can enable retraction pockets to become attached to the medial attic wall by fibrous adhesions and Akyildiz *et al.*, (1993) showed that there is papillary ingrowth of squamous epithelium in retraction pockets. These two findings reinforce the premise that persistent retraction pockets can be the precursor of cholesteatoma which in turn causes erosion by adhesion to the underlying ossicles and mastoid bone and labyrinthine capsule.

Jensen *et al.*, (1993) showed that considerably increased gas pressure, amounting to 1,200 mb (range 500 to 2,100 mb) [900 mm Hg (375-1575)] was necessary to rupture normal TM's postmortem and 99% of the perforations were located in the pars tensa. The pressure required to rupture the membrane diminished with increasing age. However, Sade (1993a/b) found that minimal changes amounting to only a few millimetres of water caused retraction or ballooning of the atrophic tympanic membrane.

Knight *et al.*, (1993) measured middle ear pressure differences during upper respiratory tract infections, and reported between 6.5 mb [4.9 mm Hg] and -14 mb [-10.5 mm Hg] mean -1.3 mb [-0.98 mm Hg] in his findings. These pressures were consistent with intermittent "dysfunction" of the Eustachian tube and gradual middle ear gas absorption.

Most, if not all, micro-organisms produce some gases during their growth cycle, depending upon the constituents of the growth medium. In addition, most micro-organisms consume some gases, including oxygen, carbon dioxide and nitrogen during their metabolic phase.

The isolates from acute otitis media cultured by me over thirty years in bacteriology at the Royal National Throat Nose and Ear Hospital, London have included: *Streptococcus pyogenes*, *Streptococcus pneumoniae* (mucoid type 3) and *Haemophilus influenzae*. By the time that many patients are referred from general practitioners to hospital out-patients and, following provisional treatment with Penicillin, Penicillin resistant *Staphylococcus aureus* is often isolated together with Gram negative bacteria. These Gram negative organisms during the chronic stages of infection, include *Pseudomonas aeruginosa*, *Proteus* and *Klebsiella* species.

Whilst many authors do not consider bacterial infection to be part of the pathogenesis of cholesteatoma (Konishi *et al.*, 1993), Gram negative bacilli, predominantly *Pseudomonas aeruginosa* and *Proteus vulgaris* have been present in 12% of the cholesteatomas in this study (personal series).

The aim of this experiment was to prepare a model in order to ascertain which, if any, of the micro-organisms commonly found in otitis media infections absorb sufficient gas during metabolism to significantly affect the pressure of the middle ear and produce invagination of the tympanic membrane.

The *in vitro* model of a closed chamber to represent the middle ear, presupposes, that middle ear ventilation and normal equilibration with atmospheric pressure during swallowing has ceased. This is presumed to occur because the Eustachian tube is thought to be "blocked" during both acute and chronic secretory otitis media. Throughout the trials, prominence is given to *Pseudomonas aeruginosa* because these bacteria are a common source of low

grade subclinical infection which can pass undetected by the immune system and which can be particularly resistant to eradication.

## A2.2 METHOD AND MATERIALS

The first model consisted of blue, opaque plastic tubing with an internal diameter of approximately 2 mm, impervious to gas. This tubing was supplied by Digitron, who supplied the pressure transducers. A length of the tubing, held in the shape of a U, was filled with distilled water from a tuberculin syringe. From the volume of water used, the length of tube equivalent to an adult middle ear space of 2 ml could be derived and this length was used in the experiment. The tube was sterilised by filling it with absolute ethyl alcohol for 10 minutes, draining and drying it in a sterile 15cm glass petri dish in a spark-proof incubator at 37°C.

## BACTERIA SUBCULTURES

Freeze dried cultures of bacteria and yeasts were obtained from the National Collection of Type Cultures and from my own collection, isolated from suppurative otitis media swabs and cholesteatomas (see Suppurative Otitis Media section). The ampoules containing freeze-dried bacteria were opened in a class 1 safety cabinet, reconstituted with 0.2 ml nutrient broth, and subcultured on the following semi-solid medium.

*Streptococcus pyogenes*, *Streptococcus pneumoniae* and Gram negative bacilli : blood agar. *Haemophilus influenzae*: chocolate agar. *Staphylococcus aureus*: nutrient agar. *Candida*: Sabouraud's agar.

## CULTURE MEDIUM USED FOR THE EXPERIMENT

Liquid broth proved unsuitable as a culture medium for estimating the gas absorption of bacteria for two reasons: (1) the nutrient in different batches of artificial medium proved too inconsistent to reproduce the results (2) during propagation, bacteria initially utilise small amounts of gas, then progressively produce copious amounts of gas. Oxoid Ltd., have developed this phenomenon, commercially, and market a rapid overnight method of detecting positive blood cultures by demonstrating this gas production. The culture medium needed for this experiment should contain nutrients which can be utilised by bacteria for propagation, at the same time these nutrients should not encourage the production of gas. The fine balance between these requirements probably exists during infection when nutriment is derived from body tissue and fluids.

Human blood, from some donors, can contain inhibitors against bacteria and incorporated in semi-solid culture medium, i.e., used for blood agar in the place of horse blood, can prove inferior and unusable. I tested my own blood, defibrinated, as a liquid culture medium for *Staphylococcus aureus* and *Escherichia coli*. These organisms were successfully propagated and recovered in subculture following incubation (37 °C) and my subsequent blood donations were therefore collected after overnight fasting and used throughout the experiment. The absence of anticoagulants and other additives presented the advantage of requiring none of these substances to be control tested.

#### BLOOD COLLECTION

10 ml of overnight fasting blood was collected in a sterile 20 ml universal glass bottle without anticoagulant. Defibrination was immediately effected by gently stirring the blood with a sterile glass rod to which fine glass projections



had been annealed. The fibrin clot collected around these projections to leave free flowing defibrinated blood. This was placed in a 37 °C incubator for two hours in order to allow the remaining polymorphic neutrophils to phagocytose any bacteria introduced during the venepuncture. The blood was then transferred to a refrigerator at 4 - 6°C and used within 3 days of collection. Immediately before use, it was oxygenated by gentle inversion for 5 minutes prior to adding bacteria or yeasts.

#### BACTERIA INOCULUM

The following organisms, commonly isolated from suppurative otitis media, were tested: *Streptococcus pyogenes*, *Streptococcus pneumoniae*\*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Proteus vulgaris*, *Klebsiella pneumoniae*\*, *Pseudomonas aeruginosa*\* and *Candida albicans*. The latter organism is regarded by some otolaryngologists as contaminatory in origin. *Candida albicans* produced a profuse growth in one cholesteatoma culture.

\*(strains which exhibited mucoid colonies at the time of testing. Mucoid strains were selected on the grounds that their mucoid tenacious capacity are most likely to clog the isthmus of the Eustachian tube and immobilise its cilia).

A standard concentration  $2 \times 10^9$  of the bacteria was prepared following their culture overnight on the previously listed semi-solid agar medium. Using a platinum wire loop, sterilised in the flame of a bunsen burner, bacteria were carefully collected from the surface of colonies without disturbing the surface of the medium and carrying over any nutrient. The bacteria were emulsified in 2 ml sterile saline in a sterile Bijou bottle, shaken to obtain an even suspension and 1 ml transferred to a sterile Brown's tube. By using a sterile Pasteur pipette

to mix the contents, sufficient bacteria or saline were added to produce a suspension matching the turbidity, viewed against a printed background, e.g., *Staphylococcus aureus* in tube Brown's tube 6 (see table). The opacity in Brown's standard tubes was produced commercially for standardising autogenous vaccines by preparing different concentrations of barium sulphate in a gel form, and hermetically sealed them as permanent preparations.

Brown's tube:	1	2	3	4	5	6	7	8	9	10	
<i>Strep. pyog.</i>	304	609	913	1217	1522	1825	2130	2434	2739	3043	millions /ml
<i>Staph. aureus</i>	379	758	1137	1516	1985	2273	2652	3031	3410	3789	..
<i>Strep. pneum.</i>	705	1411	2116	2821	3527	4232	4937	5642	6348	7053	..
<i>Esch. coli</i>	379	757	1136	1515	1894	2272	2651	3030	3408	3787	..
<i>Haem. infl.</i>	1140	2279	3419	4558	5698	6838	7977	9117	10256	11396	..

Using a sterile graduated pipette, 0.1 ml of the matched suspension of the bacteria was added to 1.8 ml saline + 0.1 ml Loeffler's methylene blue and 2 drops of undiluted formalin in a bijou bottle and thoroughly mixed. One drop of this (1 in 20) dilution was pipetted into a Neubauer counting chamber fitted with a thick plane glass coverslip. The presence of Newton's rings between the coverslip and chamber denoted absolute contact between them to produce a chamber 0.1 mm in depth. The average number of organisms in a tertiary square (1/400 sq.mm) was counted (=x) under a microscope and the number calculated per cubic millilitre:

Organisms/ml = (x) x 400 (number of squares in 1 sq mm) x 10 (depth) x 20 (dilution).

The original undiluted saline suspension of organisms was centrifuged at a slow speed (2000 rpm) until the supernatant was clear. This was discarded in 5% Hycolin disinfectant and the deposit resuspended in a volume of defibrinated blood, calculated from the known concentration of organisms /ml to produce the required number of organisms required for each species of bacteria used in the experiment.

#### A2.4 MEASURING THE GAS CONSUMED BY BACTERIA (=REDUCTION IN PRESSURE)

The external surface of the port nozzles on two transducers (Digitron P5SF) were cleaned with iso-propyl alcohol swabs and allowed to dry. Using a sterile Pasteur pipette graduated at 0.3 ml, this volume of a prepared suspension of organisms in defibrinated blood was carefully introduced into the tubing, held in the form of a U tube. Great care was taken not to contaminate the first 2cm of the inside of the tubing, in order to avoid contaminating the transducer ports . One end of the tube was attached to one of the port nozzles responding to increased pressure measured against atmospheric pressure and the other end of the tube to another transducer port nozzle responding to decreased pressures, again measured against the same atmospheric pressure. The pressure transducers were produced primarily for positive gas pressure differences, generating 2.5 mV/m bar when used over a range of 0-1000 mbar, with the blue impervious tubing. Digitron advised that these transducers could be used for negative gas pressure difference measurement.

The tubing containing the bacteria was immersed in a Grant distilled water bath at 36.5 °C along with a Digitron temperature probe. The pressure needed 45 minutes to adjust and stabilise with the rise from room temperature

and, therefore the probes were zero rated after exactly 1 hour. The water level in the bath was kept constant with a plastic shroud and floating plastic spheres and topping up with distilled water at 36.5 °C. Pressure readings were recorded at 1 hour intervals (Fig 1).

#### PRELIMINARY RESULTS USING IMPERVIOUS TUBING

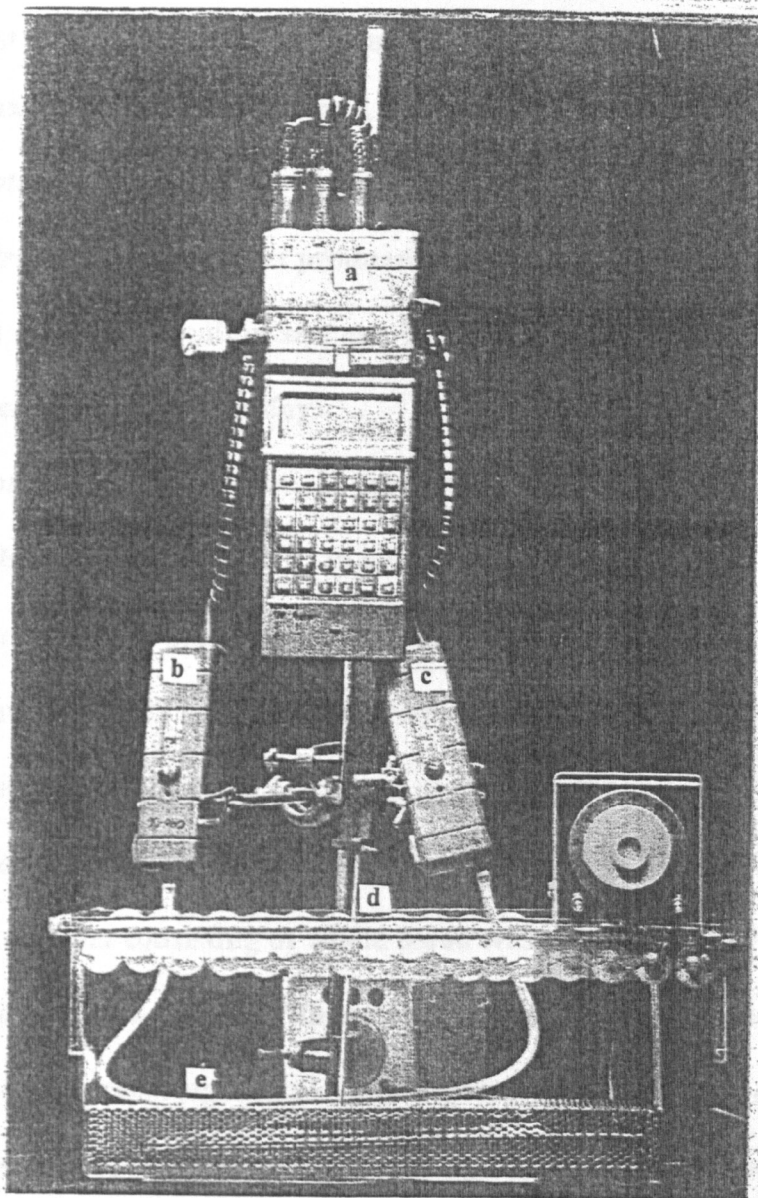
##### 8.3.4 CONTROLS

1. 0.3 ml distilled water, saline and defibrinated blood: after the initial period of of temperature stabilisation showed no further changes in pressure.
2. 0.3 ml *Pseudomonas aeruginosa*: In 6 repeat experiments, *Ps. aeruginosa* in defibrinated blood produced an average fall of 187 mb (140 mm Hg) range 183 - 191 mb although the time to reach this maximum reduction in pressure varied between experiments.

#### DISCUSSION

Compared to Knight's maximum fall of 14 mb (10.5 mm Hg) encountered in upper respiratory tract infection and Sade's results with atrophic tympanic membranes, the fall in pressure recorded indicated that the model was unphysiological. The middle ear is not a sealed impermeable chamber I decided, therefore, that the experiment should be repeated using semi-permeable tubing, to provide a model chamber more closely resembling the middle ear.

Similar bacterial suspensions in human blood were transferred to the semi-permeable silicone rubber tubing (Masterflex 96400-14)\*, impervious to water and semi-permeable to gases, and submerged in a water bath at 36.5 °C (+/- 0.2 °C), the ends of the tubing exposed 4 cm above the surface of the water were connected to the Digitron transducers as described above.



**Fig: 1**

Apparatus used to measure gas pressure

(a) Psion Organiser II recorder with three channels fitted to

(b/c) two pressure transducers and (d) temperature probe.

The tubing (e) contains my infected, defibrinated blood.

## A2.5 RESULTS USING SEMI-PERMEABLE TUBING

a. Maximum fall in gas pressure, average of six readings:

i. *Streptococcus pyogenes* 13 mb

*Streptococcus pyogenes* produced a maximum fall of 13 mb: human blood was not a suitable medium and can be inhibitory to these bacteria which were no longer viable after four days.

ii. *Streptococcus pneumoniae* 33 mb (mucoid strain)

iii. *Staphylococcus aureus* 29 mb

*Streptococcus pneumoniae* and *Staphylococcus aureus* thrived, producing maximum falls of 33 mb and 29 mb respectively.

iv. *Haemophilus influenzae* 7 mb

*Haemophilus influenzae* produced a maximum fall of 7 mb: the simple growth medium consisting of whole blood was not suitable for sustaining these fastidious bacteria for longer than 24 h. Subcultures were sterile after 48 h.

The object of preparing a 'standard' suspension of bacteria provided the option of adjusting this concentration to the optimum concentration producing gas absorption. The results obtained were considered satisfactory for the purpose of comparison however, in view of the low reduction in gas pressure produced by *Haemophilus*, the experiment was repeated using increased concentration i.e., Brown's tube 9 ( $11 \times 10^9$ ). There was no increased fall in pressure i.e., the fall depended upon the suitability of the culture medium and (my) defibrinated blood is not a suitable medium. The addition of X and V factors would probably increase propagation and entail using these factors as controls. This is



outside the scope of this experiment unless the levels of these substances (haematin and coenzyme) is determined in vivo.

v. *Proteus vulgaris* which produces copious gas in artificial culture medium, only reduced the pressure by a maximum fall of 6 mb.

vi *Klebsiella pneumoniae*: h (hours) or d (days) = Interval of time from the start of the experiment: 15h: - 56mb, 26h: - 42mb, 34h: -75mb, 55h: -34mb, 64h: -75 mb, 74h: -60mb, 92h: -76mb.

vii *Pseudomonas aeruginosa*: 5h: -33mb, 5d: -56mb, 8d: -43mb.

viii. *Candida albicans*: + 29 mb

This was the only organism, cultured in defibrinated human blood, to produce an increase in gas pressure, reaching a maximum of 29 mb.

#### A2.6 DISCUSSION

The mucoid strain of *Klebsiella pneumoniae* produced fluctuations in pressure produced by absorption of gas. Following a short lag phase, there was a maximum fall in pressure of 56 mb. The recovery in pressure from diffusion of atmospheric gas through the exposed tubing was followed by another fall, at 34 h, of 75mb. Less marked cycles of rise and fall in pressure followed, until the nutrient was exhausted.

*Pseudomonas aeruginosa* avidly absorbs oxygen and the growth pattern of these bacteria, when provided with an intermittent supply of air through the exposed semi-permeable tubing which encouraged growth, produced a recurring rise and fall in pressure until the nutrient was exhausted.

Consecutive reductions in pressure amounted to 33, 56 and finally 43 mb on day eight, followed by an extended sustained fall of 17 mb for a further 14 d.

This suggested that gas was consumed at about the same rate that it diffused through the semi-permeable tubing exposed to air at atmospheric pressure. The pressure gradually returned to zero (=atmospheric) as the bacteria perished.

The maximum fall in pressure (-56mb) was consistent (+/- 3mb) on six repeated experiments, however, the initial lag phase, which preceded reductions in pressure were not consistent. This suggested differences in the growth phase of the bacteria following inoculation of the tubing, in spite of rigorous attention given to bacteria inoculum and fasting blood samples.

The *Pseudomonas aeruginosa* selected, was a strain which proved viable in a bottle of liquid tissue culture medium (Dulbecco's modification of Eagle's medium with 10% fetal calf serum) following storage, unopened, for sixteen months at room temperature in a dark cupboard.

*Candida albicans* was the only organism to produce a positive pressure from fermentation under these conditions. For most of the time, a cholesteatoma does not induce osteoclastic activity in the bone with which it is in contact. Therefore, when cholesteatomas are colonised by *Candida albicans*, and if a positive pressure is produced, then this might contribute, in vivo, to the destructive activity of the cholesteatoma. Osteoclasts are the only cells to resorb bone and are the major effectors of the destructive action of cholesteatoma. Using animal models Chole (1993a) has shown that a rise in middle ear pressure of 4 mm Hg for 2 days results in the recruitment of osteoclasts which make a transient appearance for one to three weeks, during active bone resorption.

#### A2.7 SUMMARY

*Streptococcus pyogenes* and *Haemophilus* appeared to be poor contenders in the formation of retraction pockets whereas the mucoid strains of *Pseudomonas aeruginosa* produced significant falls in pressure and retained an extended modest negative depression. In addition, it survived without additional gas replenishment in a closed system containing a buffered pH medium for at least sixteen months. This may be of some importance because subclinical infections with *Streptococcus pneumoniae* followed by *Pseudomonas aeruginosa* are capable of producing sustained negative middle ear pressure of a sufficient level to cause serious retraction and so may be contenders in the formation of retraction pockets and cholesteatoma. In addition, some low grade infections during childhood may not provoke or elicit an immune response.

*Pseudomonas aeruginosa* is antagonistic to *Staphylococcus aureus*, therefore test mixtures of these bacteria only resulted in recovery of *Pseudomonas aeruginosa*.

Fluctuations in pressure during acute infection must impose considerable strain on the elasticity of the tympanic membrane besides questioning the role of ventilation tubes. On the one hand, the benefit of TM ventilation tubes in order to drain exudate and compensate pressure differences with atmospheric during the absence of Eustachian tube ventilation cannot be underrated. On the other hand, their employment without adequate antibiotic cover to eradicate the infecting bacteria may help to feed those bacteria with gases necessary for their survival and propagation. It could even be suggested that the closed environment of the middle ear in serous otitis acts as a defence mechanism. The presence of glue ear exudate which is invariably sterile and could fill the middle

ear cavity would starve many bacteria (e.g., *Pseudomonas aeruginosa*) of their essential gas requirements.

My present model does not allow for the complete exclusion of gas, a small quantity of which is always present in the sensors. In addition, the proportions of gases in atmospheric gas is different to middle ear gas (see Eustachian tube section).

### APPENDIX 3

*Pseudomonas aeruginosa*: rod shaped 1.5-3 $\mu$  X 0.5 $\mu$  Gram negative bacillus . Non-sporing, strict aerobe. Some strains are mucoid. Produces slime in static cultures containing potassium gluconate. Motile 1-3 polar flagella, presence of oxygen or arginine is necessary for motility. Produces ammonia from arginine and oxidises gluconate\* to 2-ketogluconate, KCN resistant. Forms hydrocyanic acid. Ferments glucose, arabinose, xylose and glycerol, some form a laevan from sucrose, all strains are catalase and oxidase positive. Indole positive, liquifies gelatin and peptonizes milk. Nitrites demonstrated after one day but nitrite itself is reduced after several days. On solid media, the organisms produce iridescent patches with a metallic sheen. Not heat resistant, max 55°C/1h. Utilises citrate as sole source of carbon. A green pyocyanin pigment is formed which, unlike *Ps.fluorescens*, is soluble in chloroform and water. Greenish-yellow fluorescent pigment formed in the presence of phosphate is soluble in water, not in chloroform. Somatic antigen, twelve groups identified by precipitation and O agglutination. Habitat: intestinal canal, skin, sewerage. Pathogenicity depends upon individual strain, pathogenic for sugar-cane, tobacco and lettuce (Elrod et al 1942). \*Gluconate: Inoculate (4% potassium or sodium) gluconate broth, incubate 2 days, add 1ml Benedict's qualitative solution, boil, brown precipitate: +ve. Controls +ve: *Kleb.aerogenes*, -ve: *Esch.coli* (*Ps.aeruginosa* its characterization and identification Haynes WC: *J.Gen.Microbiol*, 5, 939 1951).

Pigment production: Incubate at 37 °C 24-48h, room temp 72h, non-pigmented strains are not uncommon.

Quinolone antibiotics (e.g., Ciprofloxacin) are currently the therapeutic choice.

### **Pseudomonas aeruginosa Type Identification**

**Four strains, isolated from cholesteatomas and with differences in pigment production, between October 1994 and January 1995 were phage by the Public Health Laboratory Service as follows:**

<b>O type</b>	<b>Phage type</b>
(1) 06	3—1—23—3—
(2) 06	-3-2-3—3-3-32—
(3) 06	3—3—1—
(4) 06	3-1—3—33-1-3—

**Report received with the following comments: (2) is distinct, the other 3 may in fact be the same strain, would you like this confirmed with DNA fingerprinting?**

**(Reply to PHLS thanking them for their kind offer)**

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